LYSOSOMAL FRACTIONS FROM TRANSITIONAL EPITHELIUM

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ABSTRACT

Histochemical data suggested that the so called lipoid granules of transitional epithelium in some species are equivalent to lysosomes. Scrapings of bovine and canine transitional epithelium were subjected to differential centrifugation to confirm this identification biochemically. Fractions of rat liver, the classic source of lysosomes, were also prepared by the same methods to compare with the fractions obtained from urinary epithelium. In contrast to rat liver, uroepithelial fractions with a high relative specific activity for hydrolases were sedimented before the heavy mitochondria. Microscopically, these fractions contained the highest proportion of lipoid granules. The size and sedimentation characteristics of lysosomes from transitional epithelium more closely resembled those of lysosomes derived from rat kidney than those isolated from liver.

INTRODUCTION

The centrifugal separation and characterization of a class of subcellular particles from rat liver by de Duve *et al.* (6) has led to the widespread experimental investigation of these particles which have been called lysosomes. Although biochemical, histochemical, and electron microscope studies of lysosomes have been numerous, the number of investigations in which lysosomes have been separated from other cellular constituents and characterized enzymatically have been few.

The presence of lipid-rich droplets in transitional epithelium was noted as early as 1911 by Bauch (2). Takahashi (17) coined the term "lipoid granules" for these cytoplasmic structures because he felt that they did not represent neutral lipid inclusions. Since the lipoid granules in the transitional epithelium of several species had been histochemically identified as lysosomes (9), an attempt was made to isolate them by differential centrifugation from the epithelium of species in which there was a correspondence between lipoid granules and histochemically detectable lysosomes, and to determine their enzymatic characteristics biochemically.

MATERIALS AND METHODS

Histochemistry

Lipids were demonstrated in frozen sections with Sudan black B after fixation in 10 per cent phosphate-buffered neutral formalin. Acid phosphatase activity was visualized in formol-calcium–fixed frozen sections employing the napthol AS-pararosaniline method of Barka and Anderson (1) and a modified Gomori technique (11).

Preparation of Fractions

For each of the centrifugal fractionations of bovine transitional epithelium, the urinary bladders

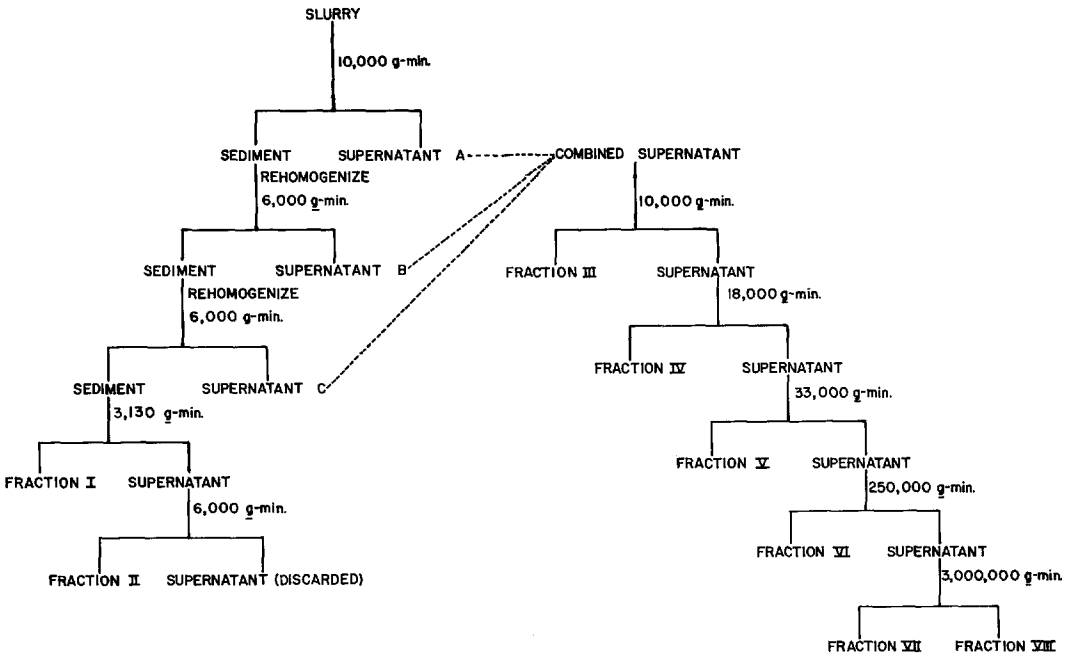

FIGURE 1 Schematic representation of the procedure utilized in the differential centrifugation of transitional epithelium and rat liver. The term "*g*-min." is that advocated and utilized by de Duve and Berthet (*5*) to enhance the reproducibility of results. It is defined as the time integral of the field prevailing in the middle of the centrifugal fluid.

of 4 freshly slaughtered steers were opened and pinned out with the urinary surface upward. The transitional epithelium was scraped with a razor blade and collected in tared beakers chilled in ice. Each beaker contained 2.0 ml of an isolation medium which was composed of 0.25 M sucrose and 0.001 M ethylenediaminetetraacetate (EDTA) and whose pH was adjusted to 7.4. The pooled scrapings provided 4 to 7 gm of tissue. The tissue was further diluted to 5 times the original weight of the tissue in grams. In the cold room the tissue was homogenized in a glass tube with a motor-driven Teflon pestle by 10 passes of the pestle. The resultant slurry was centrifuged in a refrigerated Serval preparative centrifuge (Model RC II) maintained at −2°C using rotor No. SS 34 (R_{max} = 10.8 cm, R_{min} = 4.5 cm).

In initial experiments, the fractionation scheme of de Duve *et al.* (6) for rat liver was followed. With this scheme hydrolase-rich fractions from bovine transitional epithelium were sedimented before those with cytochrome oxidase activity. Because of this and the fact that the lipoid granules, which were histochemically identified as lysosomes, ranged up to 3 μ in diameter, a modified fractionation scheme was developed which finally resulted in 8 fractions. This scheme is shown schematically in Fig. 1.

The sediment resulting from the original homogenization was rehomogenized 2 times by passing the pestle through the tissue 5 times after diluting with the same amount of medium in order to insure complete homogenization of remaining intact cells. The "combined supernatant" was diluted with the isolation medium to a final volume in ml equal to 10 times the original wet weight of the tissue in grams. Fraction I was washed once by resuspending the sediment with a single pass of the pestle after diluting with 10 ml of isolation medium and then resedimenting by the same field-time as used for its initial isolation. To remove nuclear contamination, Fractions II to V were resuspended as above and centrifuged by 3,130 *g*-min.[1] with the sediment being discarded. The supernatants were then centrifuged by the same field-time used to obtain the given fraction. The given fraction was then washed once, again employing the same field-time for resedimentation and discarding the wash-supernatant. Fraction VI was washed once.

Each of the fractions was examined microscopically with reduced illumination. The unstained specimen was prepared by mixing a small quantity of sediment with 2 or 3 drops of isolation medium.

In the study of canine transitional epithelium, the same separation scheme was followed as outlined

[1] See legend to Fig. 1.

above. The urinary bladders were removed from 4 dogs under Nembutal anesthesia. The pooled scraping provided 0.8 gm of epithelium.

Rat liver was also fractionated by the revised scheme used for the transitional epithelium.

Biochemical Enzyme Assays

ACID PHOSPHATASE: Determinations were performed according to the method described in Hawk, Oser, and Summerson (8).

β-GLUCURONIDASE: The enzyme was assayed by a slight modification of the method detailed in Sigma Technical Bulletin No. 105 using 0.0001 M phenolphthalein glucuronide (Sigma) in the incubation mixture.

CYTOCHROME OXIDASE: This enzyme was measured polarographically in a G.M.E. Oxygraph. The incubation mixture consisted of 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.3.), 0.2 ml of 6×10^{-4} M cytochrome *c*, and 0.3 ml of 0.25 M as-

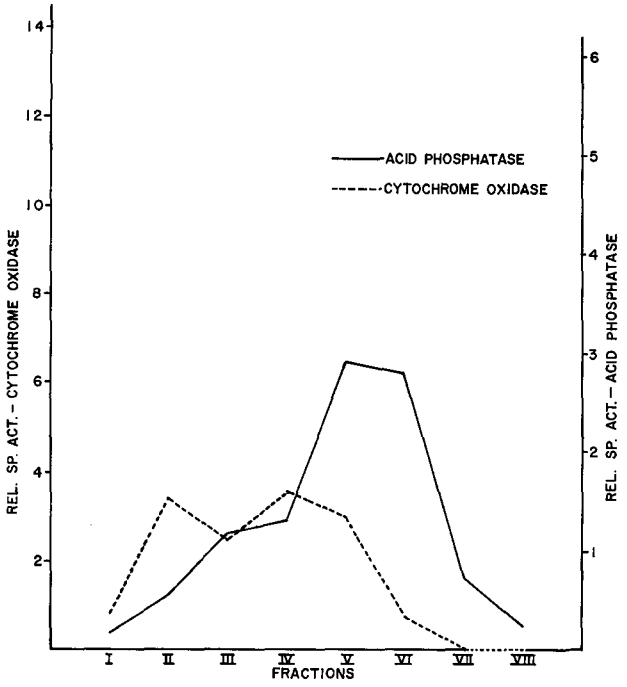

FIGURE 2 Rat liver. Sedimentation patterns formed by the simultaneous plotting of the relative specific activities (*Rel. Sp. Act.*) for cytochrome oxidase and acid phosphatase in fractions obtained by differential centrifugation.

TABLE I

A Comparison between the Relative Specific Enzyme Activities in Fractions Obtained by the Differential Centrifugation of Canine Transitional Epithelium and Those Obtained from Rat Liver under the Same Conditions

Fraction		I	II	III	IV	V	VI	VII	VIII
Canine transitional epithelium	Cytochrome oxidase	0.49	0.00	2.46	1.69	9.56	0.95	0.37	0.39
	Acid phosphatase	0.11	1.98	3.52	2.86	2.56	0.68	0.89	0.39
Rat liver	Cytochrome oxidase	0.85	3.42	2.48	3.53	2.96	0.74	0.00	0.00
	Acid phosphatase	0.19	0.55	1.19	1.33	2.91	2.81	0.73	0.23

FIGURE 3 Frozen section of bovine transitional epithelium stained with Sudan black B. Moderate to intensely sudanophilic lipoid granules are visible around the nuclei. × 1100.

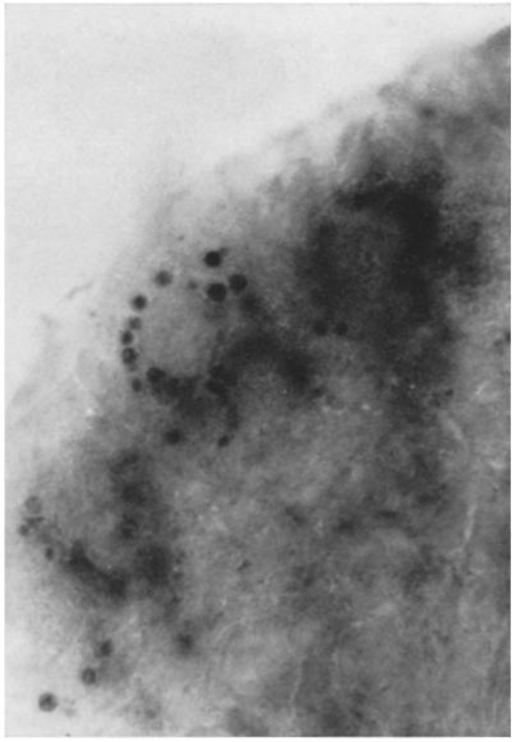

FIGURE 4 Bovine transitional epithelium incubated for acid phosphatase activity (1). Enzymatic activity is present in paranuclear droplets which correspond in size, number, and distribution to the lipoid granules of Fig. 3. × 1200.

corbic acid. Added to this was 0.1 ml of 0.04 M EDTA to minimize autooxidation. The reaction was started by the addition of 0.1 ml of sample.

Protein was determined by the method of Lowry *et al.* (12).

All enzyme assays were performed on fractions which were stored overnight at 2°C.

RESULTS

Rat Liver

The sedimentation patterns obtained for the activity of acid phosphatase and cytochrome oxidase resembled those obtained by de Duve *et al.* (6) who employed 5 fractions. Fraction I consisted of isolated nuclei with contamination by red cells and some mitochondria. Fractions II to VI all contained slightly refractile particles of approximately the same size within a given fraction. The particle size, however, did decrease gradually from Fraction II to Fraction VI. Fraction VII showed no visible particulates under the microscope, and grossly the sediment had the typical red jelly-like appearance of the microsomal fraction.

In Fig. 2 and Table I it can be seen that the highest relative specific activities for cytochrome oxidase were present in fractions sedimenting before fractions with the highest relative specific activity for acid phosphatase. The relative specific activity for each fraction was obtained by dividing the per cent of the total enzyme activity by the per cent of the total protein in that fraction. The total enzyme activity and the total protein were determined by the summation of the respective values for each of the fractions. The highest relative specific activities for acid phosphatase were present in Fractions V and VI. Fraction VI was sedimented by a field-time equivalent to that used by de Duve *et al.* (6) in the preparation of the light mitochondrial or lysosomal fraction of rat liver.

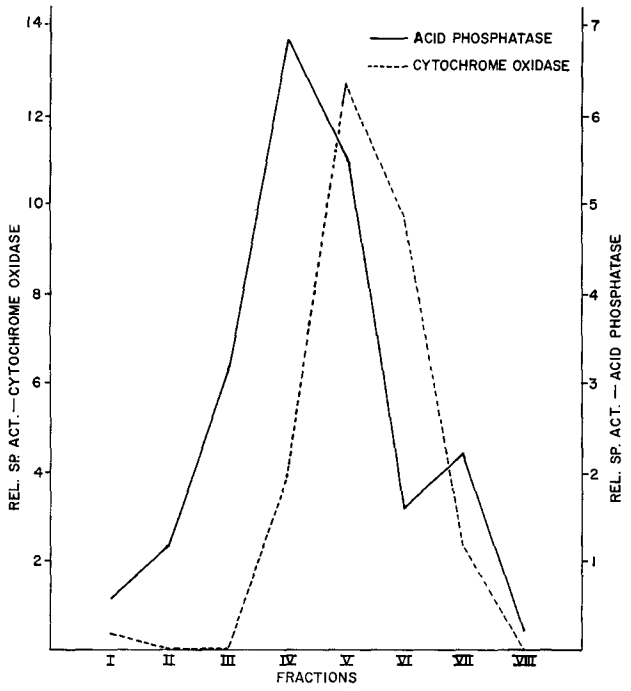

FIGURE 5 Bovine transitional epithelium. Altered sedimentation patterns of cytochrome oxidase and acid phosphatase obtained with epithelial scrapings using the same fractionation scheme employed for rat liver (Fig. 2).

Bovine Transitional Epithelium

The lipoid granules of the bovine epithelium range up to 3 μ in diameter and are distributed as paranuclear rings[2] (Fig. 3). Their number and average size increase as one proceeds from basal to surface cells. In unstained sections, a yellow-brown pigment can be demonstrated in them. Sections incubated for acid phosphatase activity (Fig. 4) reveal that there is a correspondence in size, number, and distribution between lipoid granules and histochemically demonstrable lysosomes.

When fractions which were prepared by differential centrifugation were examined microscopically, the larger size, pigment, and higher refractility of the lipoid granules permitted their ready differentiation from mitochondria which were only slightly refractile. Fraction I was found to consist mainly of isolated nuclei and rare intact cells. Fractions II to V contained lipoid granules whose average size decreased from Fraction II to V. In addition, these fractions contained mitochondria, the proportion of these increasing from Fraction II to V. Fraction VI showed homogeneous fields of slightly refractile particles.

Fig. 5 shows that in contrast to rat liver the highest relative specific activity for acid phosphatase was present in Fraction IV. Fractions II to IV always displayed higher relative specific activities for acid phosphatase than for cytochrome oxidase, and it was these fractions in which the highest proportion of lipoid granules was found. Fraction VI, sedimented under the same conditions as Fraction VI of rat liver, showed relatively high cytochrome oxidase activity and low acid phosphatase activity.

Studies comparing β-glucuronidase with cytochrome oxidase activity in bovine transitional epithelium were made prior to the development of the final fractionation scheme consisting of 8 fractions. Fraction A in Fig. 6, however, was sedimented by 6000 g-min. and can be expected to contain the particulates sedimented in Fractions I and II of the final fractionation scheme. Fraction B, likewise, can be considered equivalent to Fractions III and IV of the 8 fraction scheme since it was sedimented by 18000 g-min. Fractions C, D, E, and F were obtained under conditions that made them equivalent to Fractions V, VI, VII and VIII, respectively. Fig. 6 demonstrates

[2] Data on further histochemical properties of lipoid granules in transitional epithelium will be published elsewhere.

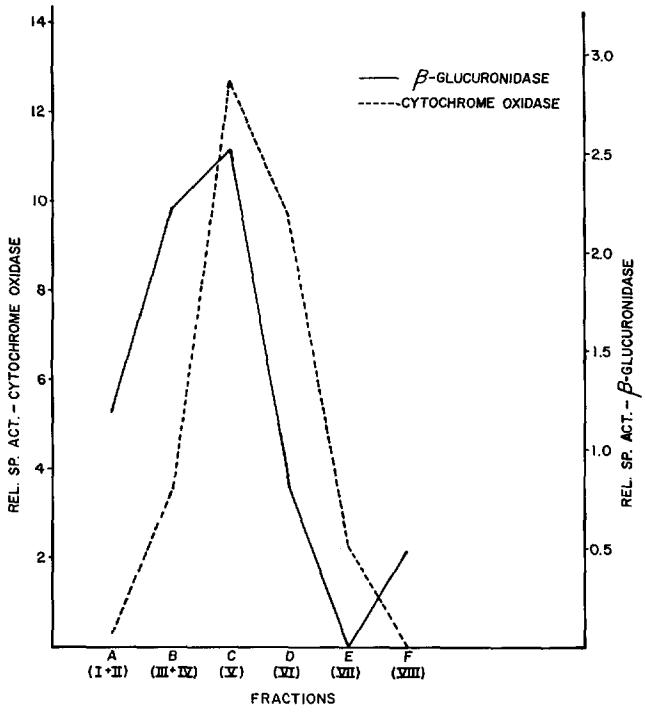

FIGURE 6 Bovine transitional epithelium. Sedimentation patterns of cytochrome oxidase and β-glucuronidase.

that high relative specific activities for β-glucuronidase and low specific activities for cytochrome oxidase were present in Fractions A (I and II) and B (III and IV). Fraction C (V) contained high relative specific activities for both enzymes. It will be recalled that microscopically this fraction contained the smallest lipoid granules and a high proportion of mitochondria. Fraction D (VI) which microscopically consisted of homogeneous fields of slightly refractile mitochondria possessed a realtively high cytochrome oxidase activity and a relatively low β-glucuronidase activity.

Canine Transitional Epithelium

In the dog, there again is a correspondence in size, number, and distribution between lipoid granules (Fig. 7) and histochemically detectable lysosomes (Fig. 8). The granules are non-pigmented in this species and reach a maximum diameter of 6 μ. Larger ones demonstrate evidence of internal structure discernible even at the light microscopic level.

In Table I, a comparison is made between the data obtained with canine transitional epithelium and rat liver. In the transitional epithelium of the dog the highest relative specific activities for acid phosphatase were found in Fractions III and IV which were sedimented before Fraction V. It was the latter which possessed the highest relative specific activity for cytochrome oxidase.

DISCUSSION

Lysosomes were first described and defined as a distinct class of subcellular particles in rat liver (6). In the parenchymal cells of this organ they have an average diameter of 0.4 μ and sediment between mitochondria and microsomes. Later, the presence of lysosome-like particles in rat brain was first suggested in another biochemical study because hydrolytic enzymes were bound to particulate components within which they were not fully active unless a lysing agent (Triton X-100) was added (3). However, they could not be separated from mitochondria. A concentration of acid phosphatase in a "light mitochondrial" fraction of rat brain at nearly three times its concentration in the total homogenate has also been reported (10).

Wattiaux (19) has studied the sedimentation patterns of several hydrolases and cytochrome oxidase after differential centrifugation and density-gradient centrifugation of homogenized HeLa

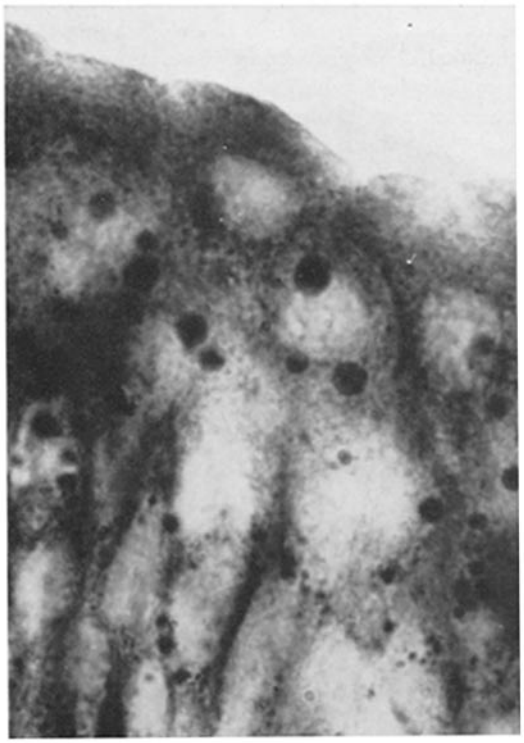

FIGURE 7 Canine transitional epithelium stained with Sudan black B. In this species the lipoid granules are also paranuclear, but reach a maximum size of 6 μ. $\times$ 1200.

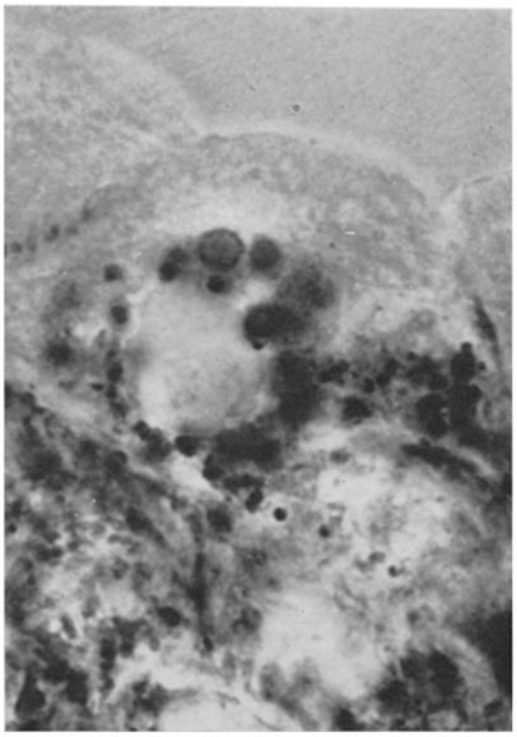

FIGURE 8 Canine transitional epithelium incubated for acid phosphatase activity (11). The lipoid granules of Fig. 7 all appear to contain enzymatic activity. $\times$ 1600.

cells. It was concluded that the lysosomes of these cells were similar to those of rat liver. Tappel *et al.* (18) in an analysis of skeletal muscle have noted that hydrolase activity sedimented in the mitochondrial fraction, in fractions sedimenting between mitochondria and microsomes, and with the microsomal fraction. No histochemical analysis of the tissue was made and the morphological composition of the fractions was not examined. On the other hand, Rahman (15) has prepared fractions of rat thymus by density-gradient centrifugation and has examined these biochemically and electron microscopically. Electron-opaque bodies with a mean diameter of 0.25 μ were found in highest concentration in fractions high in acid phosphatase, while fractions rich in cytochrome oxidase consisted mostly of mitochondria. Thus, in the studies cited above, the lysosomes of several tissues have exhibited size and sedimentation characteristics similar to those of rat liver.

In the present study, the separation of fractions from bovine and canine transitional epithelium containing high relative specific activity for acid phosphatase and β-glucuronidase, low cytochrome oxidase, and large numbers of refractile granules of the same size range as the lipoid granules of these species substantiates the histochemical identification of these granules as lysosomes. The presence of lysosomes in transitional epithelium has, in passing, been previously suggested from histochemical data (14). The lysosomal fractions of transitional epithelium in the species studied here have been shown to differ from those of rat liver in the same study by a direct comparison employing identical methods of preparation. The only other isolated lysosomal fractions which have been shown to have size and sedimentation characteristics different from rat liver are those obtained from rat kidney (16). Droplets up to 6 μ in diameter have been separated from rat kidney which have high concentrations of acid phosphatase, ribonuclease, deoxyribonuclease, β-glucuronidase and cathepsin, and which sediment between nuclei and mitochondria.

Morphological analysis of the fractions obtained from the bovine epithelium was relatively simple because the lysosomes range up to 3 μ in diameter and contain pigment. Nuclei were obvious because of their large size and lack of pigment. Mitochondria were only slightly refractile and non-pigmented. Only the studies of Straus on rat kidney (16), of Novikoff *et al.* on the liver (13), and of Rahman on the thymus (15) have involved a morphological analysis of lysosomal fractions from other tissues. In addition to morphological analysis, the present study employed the mitochondrial enzyme, cytochrome oxidase, to determine the degree of mitochondrial contamination of the lysosomal fractions. Although Koenig and Jibril (10) speak of obtaining a "light mitochondrial" fraction from rat brain with a hydrolase concentration of three times that in the total homogenate, they make no mention of morphological confirmation of the content of the fractions or determination of cytochrome oxidase activity. Other biochemical studies (4, 7) have only established that lysosomes are present in various organs by inference, since the only criteria of identification have been the presence of sedimentable hydrolase activity and/or the presence of latent hydrolase activity.

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