Characterization of Argentosomes, a Type of Secondary Lysosomes, and the Sub-organellar Distribution of a Lysosomotropic Basic Compound

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Secondary lysosomes (argentosomes) were isolated by centrifugation in a discontinuous sucrose density gradient from livers of rats administered colloidal silver. Compared to the crude homogenate, the purities of the argentosome preparations were 17.4- and 18.5-fold in terms of acid phosphatase and *N*-acetyl- β -glucosaminidase activities, respectively. By lipid analysis, the argentosomes were shown to have intermediate properties between normal lysosomes and tritosomes with regard to the contents of triglyceride and cholesterol. The phospholipid content in the argentosomes was also different from that in these two organelles. The cross-point of argentosomes shifted more to the acidic side than that of normal lysosomes.

The data on the binding of tritiated *p*-biphenylmethyl-(*dl*-trotyl- α -tropinium)bromide ($[^3\text{H}]\text{BTTB}$) to argentosomes indicated that the degree of binding and/or incorporation of this basic compound to the organelles was much higher than that to normal lysosomes. These results suggested that the distribution of BTTB on or within argentosomes might be under the control of the surface charge of the argentosomal membranes.

Keywords — argentosome; secondary lysosome; lysosome; tritosome; *p*-biphenylmethyl-(*dl*-trotyl- α -tropinium)bromide (BTTB); organellar membrane; cross-point

Introduction

Lysosomes are morphologically heterogeneous, and a variety of methods have been proposed for the isolation of rat liver lysosomes.^{1,2)} In general, lysosomes can be separated from mitochondria and peroxisomes on the basis of the density of rat lysosomes after the injection of such agents as Triton WR-1339 and Dextrane which alter the native lysosomes. When colloidal silver is injected into rats, it is incorporated into hepatocytes by endocytosis. Phagosomes are thus formed and these vesicles then fuse with the primary lysosomes, giving rise to secondary lysosomes called argentosomes³⁾ whose density is much higher than that of native lysosomes.

p-Biphenylmethyl-(*dl*-trotyl- α -tropinium)bromide (BTTB), a quaternary ammonium derivative of tropane alkaloids, is one of the lysosomotropic basic compounds. We recently reported⁴⁾ that BTTB is bound to lysosomal membranes of rat liver with a high affinity.

Little is known about the biochemical properties of the argentosomes and the distribution of

BTTB within the organelles. The present study was carried out in order to clarify the biochemical properties of rat argentosomes and the distribution of BTTB within the organelles.

Materials and Methods

Animals and Drugs — Male Wistar rats, weighing approximately 200 g, were used. ^3H -Labeled BTTB ($[^3\text{H}]\text{BTTB}$, the ^3H was introduced into the 3rd position of the biphenyl moiety) with a specific activity of 0.149 or 0.354 mCi/mg in 0.9% NaCl was used throughout the experiments. Colloidal silver (Art 7447, content of Ag was 8%, pH 7—9, as AgNO_3 : 0.05%, water: 7%) was purchased from E. Merk (Germany). This was used in the form of emulsion of 60 mg/ml in 0.9% NaCl.

Preparation of Subcellular Fractions — Rats were killed by decapitation and the livers were quickly removed, immersed in cold 0.25 M sucrose and weighed. Normal lysosomes were isolated according to the method of Kato⁵⁾ and mitochondria, according to Parson *et al.*⁶⁾ Mi-

Fig. 1. Flow Chart for the Preparation of Argentosomes

osomes were obtained by the method of de Duve *et al.*⁷⁾ Tritosomes were prepared as described previously.⁸⁾

Preparation of Argentosomes from Rat Livers — A daily dose of 72 mg/kg of colloidal silver was injected intraperitoneally for 3 d in two portions daily into rats (180–200 g).³⁾ The rats were starved for 12 h before sacrifice and [³H]BTTB (59.6 μ Ci/ 100 g b.w.) was then given intraperitoneally 1 h before sacrifice. Cell fractionation was initiated by homogenization of the liver in 9 vol of 0.25 M sucrose. A sample of the homogenate was used for the determination of lysosomal enzyme activities and radioactivity. Procedures for the preparation of the argentosomes is summarized in Fig. 1.

Determination of Cross-Points of Partition in Aqueous Polymer Two Phase System — Cross-Points were estimated by partition in aqueous polymer two-phase systems. The phase systems used: System I: 6% (w/w) Dextran T-500, 6% (w/w) polyethyleneglycol 4000, 250 mmol sucrose/kg, 5 mmol sodium phosphate or sodium citrate buffer/kg and 100 mmol NaCl/kg. System II: The same composition as in system I but with 50 mmol Na₂SO₄/kg instead of 100 mmol NaCl/kg.

The systems were allowed to equilibrate at 4 °C until they were thoroughly separated. Ten grams each of system I and II were poured into calibrated test tubes, to which 0.1 ml of sample

was added. They were mixed by inverting the test tubes gently several times and allowed to settle for 50 min after which time, 2.0 ml of the top phase was carefully pipetted from the system to each tube. The activity of each marker enzyme for subcellular organelles was assayed and the level of the activity in the top phase was expressed as percent of the total activity in the tube. The pH of the remaining phase was measured. No significant decrease in the recoveries of any of the enzymes assayed in the polymer system tested was observed in the present experiment.

Enzyme Assays — Acid phosphatase (acid Pase, EC 3.1.3.2) activity was determined according to the method of Appelmans *et al.*⁹⁾ Glucose-6-phosphatase (EC 3.1.3.9) activity was determined by the method of Swanson.¹⁰⁾ The liberated inorganic phosphate was measured by the method of Lindberg and Ernster.¹¹⁾ In the case of the cross-partition method, the acid Pase activity was determined according to the method of Bessey *et al.*¹²⁾ N-Acetyl- β -glucosaminidase (NAG, EC 3.2.1.30) activity was determined according to the method of Walker *et al.*¹³⁾ and the monoamine oxidase activity was determined by the method of Turski *et al.*¹⁴⁾

Determination of Contents of Lipid and Protein, and Radioactivity — Triglyceride was determined according to the method of Handel *et al.*¹⁵⁾ with modification by Ishii *et al.*¹⁶⁾ Contents of cholesterol and phospholipid were determined by the methods of Kitamura¹⁷⁾ and Matsuzawa *et al.*¹⁸⁾ respectively. Protein content was determined by the method of Lowry.¹⁹⁾ The [³H]BTTB radioactivity was measured in an Aloka liquid scintillation counter (Aloka LSC-903). Each sample (0.1 ml) was added to 10 ml of a dioxane counting scintillation fluid for counting.

Statistical Analysis — The results were analyzed for statistical significance by the Student's *t*-test.

Results and Discussion

Purity of the Argentosomes

The purity of the dark sediment fraction (argentosomes) was ascertained by determining

TABLE I. Specific Activities of Marker Enzymes in Argentosomes, Homogenate and Other Subcellular Fractions from Rat Liver

	Acid Pase	Monoamine oxidase	Glucose-6-phosphatase
Homogenate	0.013 ± 0.003 (1.0)	4.36 ± 0.81 (1.0)	0.016 ± 0.002 (1.0)
Argentosomes	0.226 ± 0.050 (17.4)	0.88 ± 0.09 (0.2)	0.011 ± 0.003 (0.7)
Mitochondria	0.028 ± 0.003 (2.2)	31.07 ± 2.01 (7.1)	0.033 ± 0.007 (2.1)
Microsomes	0.013 ± 0.002 (1.0)	0.63 ± 0.20 (0.1)	0.186 ± 0.008 (11.6)

Each value is given as the mean $\pm$ S.D. All enzyme activities are expressed in units per mg protein per min. Values in parentheses are relative specific activities (specific activity found in fraction/specific activity in the homogenate).

marker enzymes. Table I shows the specific activities of various enzymes found in the subcellular fraction.

The relative specific activity of acid Pase, a marker enzyme of lysosomes, was 17.4 as compared with the crude homogenate. The values for mitochondria and microsomes were 2.2 and 1.0, respectively. The relative specific activity of monoamine oxidase was 0.20, indicating 2.8% mitochondrial contamination in the argentosome preparation.

On the other hand, glucose-6-phosphate, a microsomal enzyme, had a relative specific activity of 0.70 as compared with the homogenate. This indicated a 5.7% microsomal contamination in the argentosomes and the sum of contaminations of the argentosome preparation by mitochondrial and microsomes was 8.5%. This preparation was considered to be sufficiently pure for the purpose of studying the properties of argentosomes.

Lipid Contents in Argentosomes

An analysis of the lipid compositions of the argentosomal preparation allowed a comparison of the purity of normal lysosomes and the trito-

somes. The tritosomes were purified about 27.6-fold with a yield of 15.3% (as estimated from the acid Pase activity). The sum of contaminations of the tritosome preparation by mitochondria and microsomes were 11.0% as estimated from relative specific activities. Table II shows the triglyceride, cholesterol and phospholipid contents of the normal lysosomes, argentosomes and tritosomes.

The amounts of triglyceride and cholesterol in the tritosomes were 138 and 787 μ g per mg protein, respectively and were approximately 6.1 and 12.5 times higher than those in the lysosomes, respectively. Higher concentration of cholesterol have been recognized as a characteristic feature of the plasma membrane.²⁰⁾ It has been reported that tritosomal preparations are relatively rich in phospholipids and cholesterol,²¹⁾ though no quantitative data has been reported for argentosomes. According to Henning *et al.*,²⁰⁾ the ratios of cholesterol to phospholipid are 0.3 and 0.52 for the lysosomal and tritosomal membranes, respectively. On the other hand, in the argentosomes, the amounts of triglyceride and cholesterol were 95 and 254

TABLE II. Triglyceride, Cholesterol and Phospholipid Contents of Normal Lysosomes, of Argentosomes and of Tritosomes from Rat Liver

	Lysosomes	Argentosomes (μ g/mg protein)	Tritosomes
Triglyceride	22.7 ± 0.4	95.3 ± 20.2 ^{a)}	138.4 ± 25.4 ^{b)}
Cholesterol	63.1 ± 5.3	254.4 ± 11.6 ^{b)}	787.3 ± 53.2 ^{b)}
Phospholipid	211.5 ± 11.7	119.2 ± 8.7 ^{a)}	171.4 ± 5.8 ^{b)}
C/P	0.3 ± 0.1	2.1 ± 0.3 ^{a)}	4.8 ± 0.3 ^{b)}

Each value is given as the mean $\pm$ S.D. from minimum of three experiments, C/P ratio represents the ratio of cholesterol to phospholipid. ^{a)} $p \pm 0.01$ vs. lysosomes. ^{b)} $p < 0.05$ vs. lysosomes.

Fig. 2. Cross-Points of Partition in Aqueous Polymer Two-Phase System of Argentosomes with Colloidal Silver

Argentosomes were obtained by the method of Stremmel *et al.*³⁾ The abscissa shows the final pH of each fraction. The ordinate is the percent recovery in the top phase of the total enzyme activity added. Each point is the mean $\pm$ S.D. of 3 experiments. The cross-point of the argentosomes is significantly different from that of lysosomes ($p < 0.05$). $\circ$, system I; $\bullet$, system II.

μg per mg protein, respectively, and these values were found to be intermediate between lysosomes and tritosomes. These observations agreed with those reported by Hayashi *et al.*²²⁾ The cholesterol content in the tritosomes was higher than that reported by Colbeau *et al.*²³⁾ The phospholipid content in the argentosomes was much lower than those in both lysosomes and tritosomes. Colloidal silver content was determined by atomic absorption analysis for the argentosomes which was degraded with concentrated nitric acid and the values was found to

be 39.5 ppm/g liver. This result suggested that the increased density of the argentosomes was not due to the increased triglyceride, but to the incorporated silver in these organelles.

Cross-Points of Argentosomes from Rat Liver

Figures 2 and 3 show the cross-points determined by cross-partition of the argentosomes and tritosomes, respectively.

As shown in Figs. 2a and 3a, the cross-point of normal lysosomes was pH 5.5. When the rats were treated with colloidal silver, the cross-point of the argentosomes shifted to pH 5.1 (Fig. 2b).

Fig. 3. Cross-Points of Tritosomes with Triton WR-1339

Tritosomes were prepared by the method of Trouet.⁸⁾ See legend to Fig. 2. The cross-point of the tritosomes is significantly different from that of the lysosomes ($p < 0.02$). Legends used are the same as in Fig. 2.

TABLE III. Distribution of [³H]BTTB Radioactivity, Acid Pase and NAG in Normal and Secondary Lysosomes

	Normal lysosomes		Argentosomes		Tritosomes	
	Activity	RSA ^{a)}	Activity	RSA ^{a)}	Activity	RSA ^{a)}
Radioactivity	8.36 ± 0.94	5.1	12.91 ± 1.80	10.7	39.24 ± 1.50	13.5
Acid Pase	0.26 ± 0.04	18.5	0.23 ± 0.05	17.4	0.80 ± 0.06	27.6
NAG	0.35 ± 0.12	15.8	0.44 ± 0.10	18.4	0.63 ± 0.04	32.9

Procedures for treatments are described in the Methods section. Values for enzymes and radioactivity are given as specific activity. Such value is the mean ± S.D. of three experiments. Enzyme units are presented as $\mu\text{mol Pi}$ formed for acid Pase or $\mu\text{mol } p\text{-NP}$ formed for NAG per mg protein/min. Radioactivities are presented as $\text{dpm} \times 10^{-3}/\text{mg protein}$. ^{a)} Relative specific activity (specific activity in the fraction per specific activity in the homogenate).

If the rats were treated with Triton WR-1339, the cross-point of the tritosomes became pH 5.2 (Fig. 3b).

The cross-partition in the aqueous polymer two-phase systems can be used to determine the isoelectric points not only of proteins^{24,25)} but also of subcellular organelles.²⁶⁾ The cross-points of argentosomes from rats given colloidal silver were determined by the cross-partition systems. This method has been useful in the present study since the polymers used have protective effects on the membranes of the organelles.²⁷⁾ The difference in the surface charge between argentosomes and lysosomes may be due to different contents of membrane phospholipids and sialic acids. Phospholipids are contained in lysosomal membranes and the disturbance in the structure and the composition of phospholipids may occur when tropane alkaloids react with the phospholipids. These are presumably a result of the change in the surface charge on these membranes.

Localization of Lysosomal Enzymes and [³H]BTTB Radioactivity after Injection of Colloidal Silver

To confirm the exclusive association of [³H]BTTB with lysosomes, we studied the uptake of [³H]BTTB by argentosomes purified from rat liver after the injection of the drug. The recoveries of proteins and the relative specific activities of acid Pase and NAG, and [³H]BTTB radioactivity in the argentosomes are shown in Table III.

The recovery of the total protein in the normal lysosomes was 8.5% of that of the crude homogenate. In contrast, the value for the ar-

gentosomes was 0.25%. The specific radioactivity of [³H]BTTB in the normal lysosomes was 5.1-fold as compared with that of the homogenate. The specific radioactivities of [³H]BTTB in the homogenates, from which normal lysosomes, argentosomes and tritosomes were prepared, were $1.64 \times$, $1.21 \times$ and 2.90×10^3 dpm/mg protein, respectively. In the argentosomes, it was nearly 11-fold and the specific activities of acid Pase and NAG were about 17.4- and 18.5-fold, respectively. In order to clarify whether the distribution of [³H]BTTB would be different between the normal and modified lysosomes, the ratios of the relative specific activity of the compound in the organelles to that of acid Pase were determined. These values were 0.61 in the argentosomes and 0.28 in the normal lysosomes. This drug is thus found to have a high affinity for argentosomes. When colloidal silver was added to the homogenate of normal liver and the argentosomes purified by the same procedure as for the silver-laden rats, the silver could barely be found in the sediment fractions and the specific activities of lysosomal enzymes in these fractions were not higher than those of the homogenate.

These results and our previous findings²⁸⁾ suggested that there is a clear relationship between the distribution of BTTB within argentosomes and the surface charge of the argentosomal membranes.

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