Biochemical Pharmacology, Vol. 21, pp. 477–484. Pergamon Press, 1972. Printed in Great Britain

METABOLISM OF QUATERNARY AMMONIUM COMPOUNDS—I

BINDING OF TROPANE ALKALOIDS TO RAT LIVER LYSOSOMES

YOSHIKAZU ECHIGOYA, YOSHIO MATSUMOTO, YOSHIO NAKAGAWA, TETSUYA SUGA and SHINKICHI NIINOBE

Division of Clinical Biochemistry, Tokyo College of Pharmacy, Kitashinjuku 3-20-1, Shinjuku-ku, Tokyo, Japan

(*Received* 3 *June* 1971; *accepted* 30 *July* 1971)

Abstract—After intravenous injection of three quaternary ammonium derivatives of tropane alkaloid, tritiated *p*-biphenylmethyl-(*dl*-tropyl-α-tropinium) bromide (^{3}H-BTTB), azoniaspiro-(3α-benziloyloxynortropane-8,1′-pyrrolidine) chloride (^{3}H-ABPC) and ^{14}C-labeled *N*-methylhyoscine methylsulfate (^{14}C-MHM), intracellular distribution of radioactivity was investigated in the liver of rats. Subcellular distribution of ^{3}H-BTTB was found to correspond reasonably well to that of acid phosphatase activity, a marker enzyme of lysosomes. The other two drugs were also specific to the light mitochondrial fraction on subcellular distribution. The bulk of the radioactivity in the light mitochondrial fraction was observed to bind to a particulate fraction corresponding in density to the lysosomes. It was found that ^{3}H-BTTB presented in the low density lysosomes of the liver of rats after treatment with RR-2020, a non-ionic detergent, and that the radioactivity bound to the light mitochondrial fraction was solubilized to some extent by the addition *in vitro* of nonradioactive drugs. These data indicated that the presence of these drugs in the light mitochondrial fraction was due to binding to lysosomal membranes.

METHYLATROPINE, *p*-biphenylmethyl-(*dl*-tropyl-α-tropinium) bromide (BTTB),* azoniaspiro-(3α-benziloyloxynortropane-8,1′-pyrrolidine) chloride (ABPC) and *N*-methylhyoscine methylsulfate (MHM), quaternary ammonium derivatives of tropane alkaloids, are all antispasmodic drugs, whose metabolic fate in animals has been reported by Albanus *et al.*,[1] Suga *et al.*,[2] Tamaru *et al.*[3] and Suga [4] respectively.

In all cases, it has been demonstrated that these drugs, after administration to animals, showed a marked accumulation in the liver, kidney and small intestine, followed by predominant biliary excretion by an active transport mechanism.

To date, however, the intracellular distribution of tropane alkaloids has not been reported. In the present work, the subcellular distribution of BTTB, ABPC and MHM in rat liver was investigated by differential and density gradient centrifugation, using ^{3}H- or ^{14}C-labeled compounds in order to obtain information to clarify the relationship between the metabolic fate and intracellular localization of these drugs.

* Abbreviations used: ^{3}H-BTTB, tritiated *p*-biphenylmethyl-(*dl*-tropyl-α-tropinium) bromide; ^{3}H-ABPC, tritiated azoniaspiro-(3α-benziloyloxynortropane-8,1′-pyrrolidine) chloride; ^{14}C-MHM, ^{14}C-labeled methylhyoscine methylsulfate.

MATERIALS AND METHODS

Animals and drugs. Male Wistar rats weighing 200–250 g were used. Radioactive tropane alkaloids were injected via the tail vein at a dose of 1 mg/kg body weight. Rats were injected intraperitoneally with RR-2020 (Nikko Chemical Company, Ltd.) at a dose of 80 mg/kg 3·5 days before sacrifice. RR-2020 is a non-ionic detergent, identical in structure to Triton WR-1339.

Tritiated *p*-biphenylmethyl-(*dl*-tropyl-α-tropinium) bromide (^{3}H-BTTB) was prepared according to a method described elsewhere,[2] and was labeled specifically at the C-2 position of the biphenyl ring with ^{3}H; its specific radioactivity was 80·6 mc/m-mole (0·149 mc/mg). Tritiated azoniaspiro-(3α-benziloyloxynortropane-8,1′-pyrrolidine) chloride (^{3}H-ABPC) was generously donated by Nikken-kagaku Company, Ltd.; it was generally labeled with ^{3}H and its specific radioactivity was 32·1 mc/m-mole (0·075 mc/mg). ^{14}C-labeled *N*-methylhyocine methylsulfate (^{14}C-MHM) was kindly supplied from Daiichi Pharmaceutical Company, Ltd., and was labeled specifically at the *N*-methyl position with ^{14}C; its specific radioactivity was 1·65 mc/m-mole (0·0039 mc/mg). The radiochemical purity of these drugs was certified by means of thin-layer chromatography using at least three kinds of developing solvent systems. R_f values of ^{3}H-BTTB, ^{3}H-ABPC and ^{14}C-MHM were 0·61, 0·36 and 0·26, respectively, on the ascending chromatogram using a developing solvent of *n*-butanol–acetic acid–water (4:1:5, by vol.).

Cell fractionation of rat liver. At a definite time after the rats were injected intravenously with the radioactive drugs, the liver was removed and homogenized in ice-cold 0·25 M sucrose in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was fractionated by differential centrifugation according to the method of de Duve *et al.*[5] Each fraction was used for the determination of radioactivity, enzyme activity and protein. The light mitochondrial fraction obtained by the above-mentioned method was further fractionated by density gradient centrifugation. Linear sucrose density gradients (1·10–1·27 g/ml) were used. Centrifugation was done at 30,000 rev/min (at minimum 38,200 *g*, maximum 89,600 *g*) for 3 hr in a Hitachi swinging bucket rotor RPS-65TA. After centrifugation, tubes were divided into eight fractions by means of a tube slicer. Radioactivity and acid phosphatase activity were determined in each fraction (see below).

Release of radioactivity from light mitochondrial fractions by addition in vitro *of nonradioactive drugs.* Five min after injection of radioactive tropane alkaloids (1 mg/kg), the light mitochondrial fraction was isolated as described above. Its suspension (100 mg fresh liver/ml) contained 1·28 ± 0·6 mg protein/ml. Various concentrations of nonradioactive drugs in 0·25 M sucrose (1·0 ml) were added and the mixture was allowed to stand for 30 min at 0° with gentle agitation. After centrifugation at 12,500 *g* for 20 min, the radioactivity of the supernatant was determined.

Assay methods. Radioactivity was determined with a liquid scintillation counter (Aloka LSC-501). The scintillation medium used consisted of 100 g naphthalein, 4 g 2,5-diphenyloxazole (PPO) and 0·4 g 1,4-bis-2-(4-methyl-5-phenyloxazole)-benzene (dimethyl POPOP) per liter of the mixture of 750 ml dioxane, 150 ml toluene and 100 ml methylcellosolve. Samples (0·1–0·5 ml) were added to 15 ml of the scintillator and the radioactivity was measured.

Acid phosphatase activity was determined using β-glycerophosphate as substrate,

and the liberated inorganic phosphate was measured according to the method of Lindberg and Ernster.[6] Enzyme preparations were treated with 0·5% final concentration of Triton X-100 before incubation.

Determination of the protein content was carried out by the method of Lowry *et al.*[7] with bovine serum albumin as a standard.

RESULTS

Subcellular distribution of radioactivity after intravenous injection of ^{3}H-BTTB, ^{3}H-ABPC and ^{14}C-MHM. Rats were injected intravenously with three radioactive drugs and the liver was subjected to cell fractionation 5 min later. Figure 1 shows the radioactivity in each subcellular fraction. The proportions of radioactivity found in the whole liver at this time was 26·2, 27·6 and 37·2 per cent of the injected dose for ^{3}H-BTTB, ^{3}H-ABPC and ^{14}C-MHM respectively. When rats were dosed with ^{3}H-BTTB, the highest specific radioactivity (3·29 × 10^4 dis./min/mg of protein) was found in the light mitochondrial fraction; the radioactivity in this fraction was 43 per cent of the total radioactivity incorporated in the liver (Fig. 1a). An apparent similarity in the distribution patterns was found between the radioactivity and acid phosphatase activity, a marker enzyme of lysosomes.

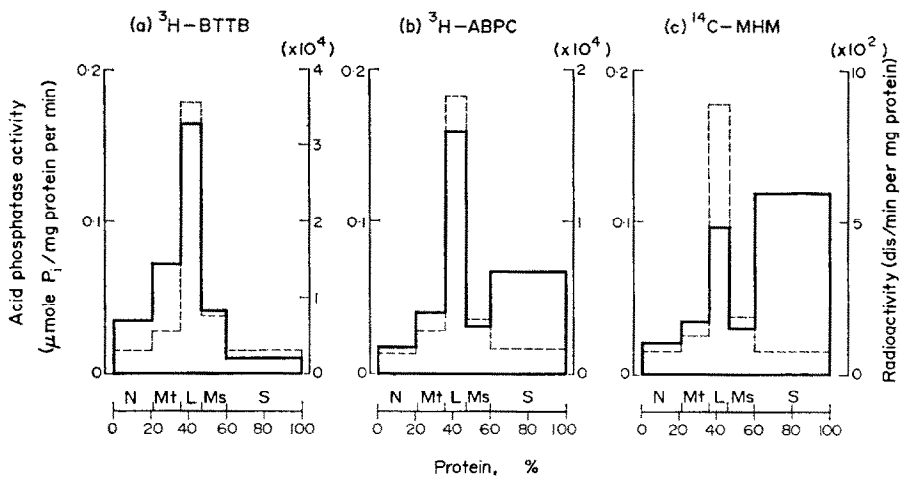

FIG. 1. Subcellular distribution of radioactivity after i.v. injection of tropane alkaloids. Rats were injected intravenously with radioactive tropane alkaloids at a concentration of 1 mg/kg. Rats were sacrificed 5 min later and subcellular fractionation of the liver was carried out according to the method of de Duve *et al.*[5] The solid line and dotted line indicate radioactivity and acid phosphatase activity respectively. N, Mt, L, Ms and S are represented as nuclear, mitochondrial, light mitochondrial, microsomal, and supernatant fraction. Values are the means of three animals.

When rats were dosed with ^{3}H-ABPC, the highest specific radioactivity (1·58 × 10^4 dis./min/mg of protein) was also found in the light mitochondrial fraction; this quantity corresponded to 29 per cent of the total radioactivity (Fig. 1b). On the other hand, a considerable amount of radioactivity (45 per cent of the total) was observed in the supernatant fraction, which contained only 11·5 per cent of the acid phosphatase activity.

Although the highest specific radioactivity (6·02 × 10^2 dis./min/mg of protein)

from ^{14}C-MHM was found in the supernatant fraction, a high specific radioactivity value ($4{\cdot}85 \times 10^2$ dis.min/mg of protein) was also observed in the light mitochondrial fraction, which corresponded to 15 per cent of the total radioactivity (Fig. 1c). In this case, too, acid phosphatase activity in the supernatant fraction was only 11 per cent of the total.

At definite times after the injection of ^{3}H-BTTB, which has the highest affinity for the light mitochondrial fraction of the three drugs employed, the distribution of radioactivity was determined in five subcellular fractions (Fig. 2). At 5 and 30 min and at 24 hr after the injection, the radioactivity in the liver was $3{\cdot}38 \times 10^6$, $2{\cdot}40 \times 10^6$ and $0{\cdot}84 \times 10^6$ dis./min/g of liver respectively.

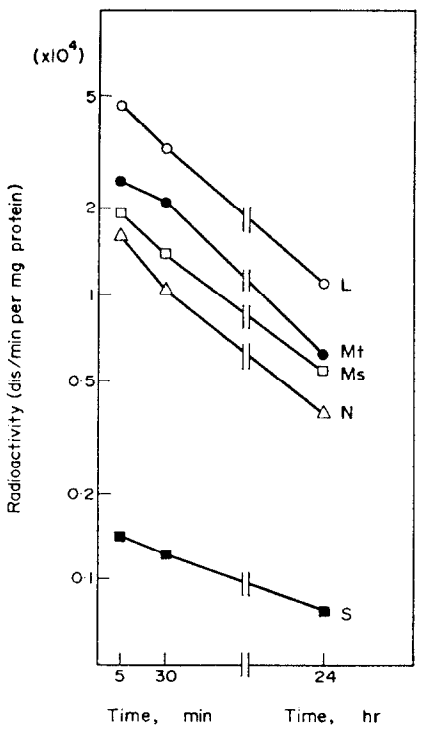

FIG. 2. Changes in radioactivity in subcellular fractions of rat liver after i.v. injection of ^{3}H-BTTB. Rats were injected intravenously with ^{3}H-BTTB at a level of 1 mg/kg (0·149 mc) and radioactivity in each subcellular fraction was determined at the indicated time intervals. N, Mt, L, Ms, and S are represented as nuclear, mitochondrial, light mitochondrial, microsomal, and supernatant fractions. Values (means of four animals) are expressed as the radioactivity (disintegrations per minute) per milligram of protein of each fraction.

In the light mitochondrial fraction, at 5 min, 30 min and 24 hr after administration, the specific radioactivity was $4{\cdot}61 \times 10^4$, $3{\cdot}21 \times 10^4$ and $1{\cdot}09 \times 10^4$ dis./min/mg of protein. The specific radioactivity in the other fractions showed similar gradual decreases. The results indicate, therefore, that the distribution ratios of the radioactivity in each fraction were approximately constant at each time after administration.

Sucrose density gradient centrifugation of the light mitochondrial fraction of rat liver

after the injection of ^{3}*H-BTTB,* ^{3}*H-ABPC and* ^{14}*C-MHM.* Rats were injected intravenously with a radioactive drug and the light mitochondrial fraction of liver was subfractionated by sucrose density gradient centrifugation; radioactivity and acid phosphatase activity in each fraction were then estimated (Fig. 3).

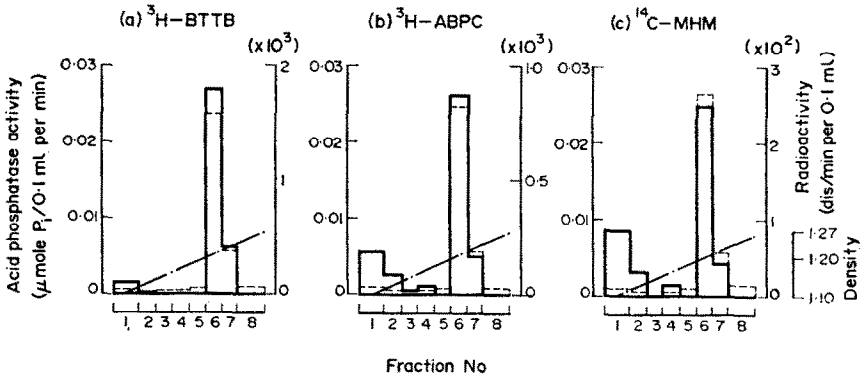

FIG. 3. Sucrose density gradient centrifugation of the light mitochondrial fraction of rat liver 5 min after the i.v. injection of radioactive tropane alkaloids. Rats were injected intravenously with radioactive tropane alkaloids and 0·5 ml of the light mitochondrial fraction was subfractionated using a linear sucrose density gradient. The centrifugation was carried out at 30,000 rev/min (at minimum 38,200 *g*, maximum 89,600 *g*) for 3 hr in a Hitachi RPS-65TA rotor. Values are means of three animals and the width of the abscissa shows the volume of each fraction.

The greatest radioactivity and acid phosphatase activity were found in fraction 6 ($1{\cdot}19 \leqq d \leqq 1{\cdot}21$). With ^{3}H-BTTB, ^{3}H-ABPC and ^{14}C-MHM, about 70, 60 and 55 per cent, respectively, of the total radioactivity was present in fractions 6 and 7 ($1{\cdot}19 \leqq d \leqq 1{\cdot}23$). These results suggest that these three drugs bind to lysosomes with considerable specificity.

In the case of ^{3}H-ABPC and ^{14}C-MHM, about 15 and 20 per cent of the total radioactivity were in the supernatant fraction (fraction 1), while only 2–4 per cent of the total acid phosphatase was observed in this fraction. These results suggest that the radioactivity in the supernatant was due to the release from the light mitochondrial fraction without disruption of the lysosomal membrane during the process of sucrose density gradient centrifugation.

It is known that the density of rat liver lysosomes decreases after the administration of Triton WR-1339.[8] In the present study, rats were injected intraperitoneally with RR-2020 (which is identical in structure to Triton WR-1339) 3·5 days before sacrifice. Five min after the same rats were injected intravenously with ^{3}H-BTTB, the light mitochondrial fraction of the liver was subfractionated by sucrose density gradient centrifugation (Fig. 4). Acid phosphatase activity was distributed broadly and, in general, migrated to lower density fractions. Changes in the distribution pattern of the radioactivity were similar to that of acid phosphatase. Radioactivity in fractions 6 and 7 declined from 71 (control) to 39 per cent (RR-2020-treated) of the total, while that in fractions 2–5 increased from 18 (control) to 50 per cent (RR-2020-treated).

Effect of addition in vitro *of nonradioactive tropane alkaloids on binding of radioactive drugs to the light mitochondrial fraction.* Five min after the intravenous injection of

radioactive tropane alkaloids, the light mitochondrial fraction which contained 1·28 ± 0·6 mg protein/ml was isolated. After the injection of ^{3}H-BTTB, ^{3}H-ABPC and ^{14}C-MHM, the specific radioactivity in these fractions was 3·57 × 10^{4}, 1·82 × 10^{4} and 5·19 × 10^{2} dis./min/mg of protein respectively.

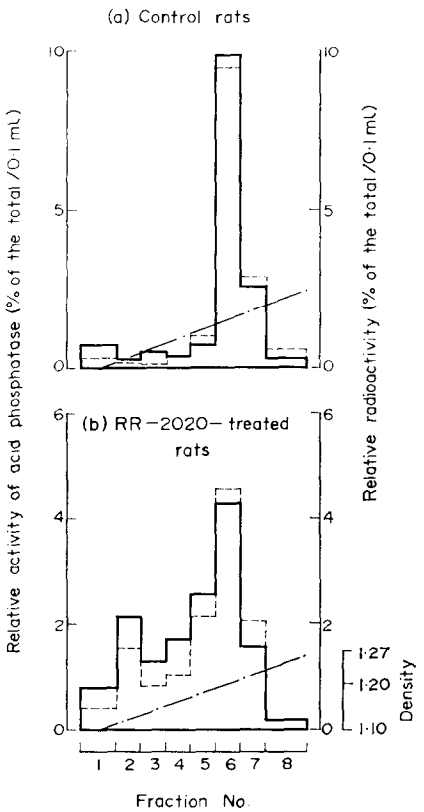

FIG. 4. Changes in distribution of the radioactivity in subfractions of the light mitochondrial fraction after i.v. injection of ^{3}H-BTTB into rats pretreated with RR-2020. Rats were injected intravenously with 1 mg/kg (0·149 mc) of ^{3}H-BTTB 3·5 days after the i.p. injection of RR-2020 (80 mg/kg) and were killed 5 min later. Sucrose density gradient centrifugation of the light mitochondrial fraction was carried out, and the radioactivity (solid line) and the acid phosphatase activity (dotted line) of each fraction were determined. Values are means of three animals.

Nonradioactive drugs were added to the suspension of the light mitochondrial fraction and the mixture was allowed to stand for 30 min at 0°. The mixture was then centrifuged and the released radioactivity in the supernatant was measured (Table 1). In the case of no addition of drugs, 5·4, 14·5 and 20·4 per cent of the radioactivity were released from the ^{3}H-BTTB, ^{3}H-ABPC and ^{14}C-MHM fractions. These findings coincide well with the radioactivity in the supernatant (fraction 1 of Fig. 3). With ^{3}H-ABPC and ^{14}C-MHM, release of the radioactivity was more pronounced than that with ^{3}H-BTTB, especially when no unlabeled drugs were added. On the other hand, with the addition of nonradioactive drugs, ratios of release were also

found to be marked with ^{3}H-ABPC and ^{14}C-MHM in comparison with ^{3}H-BTTB. The results obtained show that the binding strength of these drugs to the light mitochondrial fraction decreases as follows: ^{3}H-BTTB > ^{3}H-ABPC > ^{14}C-MHM.

TABLE 1. RELEASE OF RADIOACTIVITY FROM A PRELABELED LIGHT MITOCHONDRIAL FRACTION OF RAT LIVER BY ADDITION *in vitro* OF NONRADIOACTIVE TROPANE ALKALOIDS*

Drug administered *in vivo*	Final concn of nonradioactive drug (M)	Total radioactivity (dis./min/tube)	Released radioactivity (dis./min/tube)	Ratios of released radioactivity† (%)	Difference (%)
^{3}H-BTTB	0	27,900 ± 1800	1507 ± 112	5·4	
	10^{-4}	27,900 ± 1800	2148 ± 279	7·7	2·3
	10^{-2}	27,900 ± 1800	3543 ± 642	12·7	7·3
^{3}H-ABPC	0	14,400 ± 110	2088 ± 101	14·5	
	10^{-4}	14,400 ± 110	3082 ± 302	21·4	6·9
	10^{-2}	14,440 ± 110	4594 ± 360	31·9	17·4
^{14}C-MHM	0	396 ± 22	81 ± 4	20·4	
	10^{-4}	396 ± 22	111 ± 7	28·1	7·7
	10^{-2}	396 ± 22	132 ± 9	33·4	13·0

* Rats were injected with 1 ml/kg of radioactive tropane alkaloids and the light mitochondrial fraction was suspended in 0·25 M sucrose. Nonradioactive drugs in 0·25 M sucrose (1 ml) were added to the suspension (1 ml) and the mixture was allowed to stand for 30 min at 0° with gentle agitation, followed by centrifugation at 12,500 *g* for 20 min. The radioactivity of the supernatant was determined. Values are means ± standard deviations from five experiments.

† (Released radioactivity/total radioactivity) × 100.

DISCUSSION

The greatest radioactivity after the administration of ^{3}H-BTTB was found in the light mitochondrial fraction, and its pattern in the subcellular fractions paralleled the acid phosphatase activity, marker enzyme of lysosomes. After density gradient centrifugation, the radioactivity in the light mitochondrial fraction distributed in the fraction corresponding to the density of lysosomes, which also had the highest acid phosphatase activity. These findings indicate that in the liver ^{3}H-BTTB concentrates in lysosomes. However, attention must be paid to liver peroxisomes, which were not clearly separated from lysosomes by these methods. Wattiaux *et al.*[8] reported that in animals treated with Triton WR-1339 this agent was taken up into liver lysosomes and that Triton-loaded lysosomes decrease in density to values lower than those of intact lysosomes. When RR-2020 was used instead of Triton WR-1339 in the present experiment, the density of lysosomes was found to fall, as measured by acid phosphatase activity, and the distribution pattern of the radioactivity changed in a similar manner. From these results, it is apparent that the distribution of ^{3}H-BTTB is specific for lysosomes, but not for peroxisomes.

Although 45 and 68 per cent of the radioactivity in the case of ^{3}H-ABPC and ^{14}C-MHM were found in the supernatant fraction, significant radioactivity was also found in the light mitochondrial fraction, suggesting a similarity to ^{3}H-BTTB. When the light mitochondrial fraction of livers of rats dosed with these two drugs was

fractionated by density gradient centrifugation, the radioactivity was found in fractions identical to those containing acid phosphatase. These findings indicate that these two drugs were also concentrated in lysosomes. However, it appears that the affinity of these two drugs for lysosomes was lower than that of ^{3}H-BTTB, since 15–20 per cent of the radioactivity was liberated into the supernatant after density gradient centrifugation (Fig. 3).

Figure 2 shows that the intracellular distribution patterns were not changed with time after administration of ^{3}H-BTTB. Furthermore, as shown in Fig. 4, ^{3}H-BTTB was taken up into the low density fractions, which were assumed to contain RR-2020-loaded lysosomes. In addition, the radioactivity of the light mitochondrial fraction was released to the supernatant fraction to a different degree, indicating different extents of binding of the three drugs to the lysosomal membrane. This possibility is emphasized by the fact that the radioactivity bound to the light mitochondrial fraction was differentially solubilized by addition *in vitro* of nonradioactive drugs.

Lloyd *et al.*[9] reported that the acid bis-azo dye trypan blue, a strong protein-binder, is taken up into cells in combination with albumin, followed by a gradual accumulation in the lysosomes. Many compounds are known to concentrate in lysosomes of animal cells, for example, acridine derivatives,[10,11] polybenzenoid hydrocarbons,[12,13] neutral red,[10] antimalarial drugs,[10] and others. Allison and Young[14] described two major types of uptake of materials by the vacuolar system of the cell. The first includes particulates and dye–protein complexes. These are taken up into phagosomes which, after union with primary lysosomes, become secondary lysosomes. The second include certain basic and neutral compounds which bind to the primary lysosomes themselves.

Since the binding abilities of the three drugs used in the present work were not very strong,[2,4] they may be included in the second type according to the classification of Allison and Young.[14] In other words, it may be reasonable to consider that these drugs are taken up into liver cells in free form by a transmembrane mechanism and that thereafter they bind specifically to the lysosomal membrane.

Acknowledgement—The authors are indebted to Dr. S. Baba for his kind encouragement of this work.

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