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# Kinetics of the *in Vitro* Binding of a Quaternary Ammonium Compound (*p*-Biphenylmethyl-(*dl*-tropyl-α-tropinium)bromide (BTTB)) to Lysosomes and Other Subcellular Membranes of Rat Liver

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The extent of binding of tritiated *p*-biphenylmethyl-(*dl*-tropyl-α-tropinium)bromide ($^3$H-BTTB) to subcellular membranes was investigated by the centrifugal method. The following results were obtained.

1. The binding was apparently independent of enzymatic reactions.
2. The binding of BTTB to lysosomal membranes gave a linear Lineweaver–Burk plot with an apparent $K_m = 4.3\,\mu$M and $V_{max} = 0.25$ nmol·mg protein$^{-1}$·min$^{-1}$. *N*-Methylatropinium bromide competitively reduced the affinity of BTTB to lysosomal membranes.
3. Inhibitory effects on the binding of BTTB with lysosomal membranes were observed with choline, acetylcholine, anisotropine (quaternary ammonium compounds), quinacrine and chloroquine (tertiary amine compounds). Cetrimonium bromide (CTAB), ethidium bromide, indocyanine green, hexamethonium succinylcholine, all structurally unrelated ammonium compounds, also showed significant inhibitory effects.
4. Incubation of lysosomal membranes with increasing concentrations of indocyanine green resulted in a dose-related inhibition of the binding of $^3$H-BTTB. The binding of $^3$H-BTTB to lysosomal membranes was significantly decreased by the addition of choline at concentrations of 1 and 0.1 mM, and also by the addition of acetylcholine at a concentration of 1 mM. Methacholine and carbachol caused no change in the binding of $^3$H-BTTB at any concentration tested.

Thus, amines and quaternary ammonium compounds competitively inhibit the binding of BTTB to lysosomal membranes by virtue of their strong affinity for lysosomes rather than because of the similarity of their structures to that of BTTB, a basic compound with affinity for lysosomes.

**Keywords**——*p*-brphenylmethyl-(*dl*-tropyl-α-tropirium)bromide; quaternary ammonium compound; tertiary amine; binding property; subcellular membrane; Lineweaver–Burk plot; lysosome

*p*-Biphenylmethyl-(*dl*-tropyl-α-tropinium)bromide (BTTB), a quaternary ammonium derivative of tropane alkaloids, is an antispasmodic drug. This drug, following its administration to rats, was found to accumulate markedly in the liver, kidney and small intestine, followed by predominantly biliary excretion by an active transport mechanism.[1] In the preceding paper,[2] it was shown that BTTB could bind specifically to the lysosomes of rat liver *in vivo*. However, the binding of $^3$H-BTTB to subcellular membranes has hardly been investigated at all compared to the amount of research directed toward membrane-associated receptors for nonsteroid hormones such as epinephrine, glucagon and so on.[3–5]

In this study, the binding properties of BTTB to subcellular membranes of rat liver were examined *in vitro* by analyzing the affinity of subcellular membranes for BTTB. The effect of *N*-methylatropinium bromide, an unlabelled quaternary ammonium compound very similar in structure to BTTB, on the binding of BTTB to subcellular membranes was also examined *in vitro*. Furthermore, to clarify the mode of binding of $^3$H-BTTB to liver lysosomal membranes, the effects of various unlabelled quaternary ammonium compounds such as choline esters, betaine and so on, were examined *in vitro*.

## Materials and Methods

**Animals**——Male albino rats of Wistar strain, each weighing approximately 200 g, were obtained from Tokyo Experimental Animals (Itabashi, Tokyo).

**Materials**——Tritiated *p*-biphenylmethyl-(*dl*-tropyl-α-tropinium)bromide ($^3$H-BTTB) was prepared according to a method described elsewhere,[1] and labelled specifically at the C-2 position of the biphenyl ring with $^3$H; its specific radioactivity was 190.1 mCi/mmol (0.354 mCi/mg). Betaine, neostigmine, hexamethonium, succinylcholine, decamethonium, gallamine, *d*-tubocurarine, paraquat, chloroquine and quinacrine were purchased from Sigma (St. Louis, U.S.A.). *N*-Methylatropinium bromide, choline ester and atropine were supplied by Merck (Germany). Cetrimonium bromide (CTAB) was obtained from Eastman Kodak (U.S.A.). Anisotropine and indocyanine green were obtained from Sankyo Co., Ltd., (Tokyo) and Daiichi Seiyaku Co., Ltd., (Tokyo), respectively. Ethidium bromide was obtained from Aldrich Chemical Co., Inc.

**Preparation of Subcellular Membranes**——Rats were killed by decapitation and their livers were rapidly removed, immersed in cold 0.25 M sucrose and weighed. The partially purified lysosomes were isolated according to the method of Kato *et al.*[6] Lysosomal membranes were obtained by freezing and thawing (seven cycles) of the lysosomes. Microsomes were isolated according to the method of de Duve *et al.*[7] Microsomal membranes were isolated from the microsomes according to the method of Kreibich *et al.*[9] Preparation of the outer mitochondrial membranes was performed according to the method of Parson *et al.*[8] which is a combination of the differential $^3$H-BTTB was $10^{-4}$ M. Buffered sucrose was used as the incubation medium. After appropriate time intervals, the

**Time Course of Binding of $^3$H-BTTB to Subcellular Membranes *in Vitro***——Aliquots (0.5 ml) of subcellular membranes were incubated in test tubes with $^3$H-BTTB (0.5 ml) in a water bath at 25 °C. The final concentration of $^3$H-BTTB was $10^{-4}$ M. Buffered sucrose was used as the incubation medium. After appropriate time intevals, the incubations were terminated by the addition of $10^{-3}$ M unlabelled BTTB (6 ml) and the suspensions were centrifuged for 60 min at 100000 *g*. The binding of $^3$H-BTTB was measured in the pellet fraction.

**Estimation of Specific $^3$H-BTTB Binding to Subcellular Membranes**——Four series of tubes were designated as A, B, C, and D. To each tube in the series, a 0.1 ml sample of subcellular membrane (4.67 mg protein/ml) was added. Series A and B each received intact subcellular membrane. Series C and D each received subcellular membrane pretreated with 10.7 mM *N*-methylatropinium bromide. Next, 0.1 ml aliquots of $^3$H-BTTB ranging in concentration from 0.015 to 0.12 mM were added to all tubes in each series and 2 ml aliquots of 6.2 mM unlabelled BTTB were added to the tubes of series B and D. The reactions took place in a final volume of 3 ml following the addition of a suitable amount of 0.25 M sucrose–0.02 M tris-maleate buffer (pH 7.2).

Series A and C represent total binding, and series B, and D, non-specific binding. The specific binding of $^3$H-BTTB was defined as the difference between the total and non-specific bindings. All tubes (final vol. 3 ml) were incubated for 30 min at 25 °C in a shaking water bath. After incubation, the tubes were placed in an ice bath. At this point, 5 ml of the above ice-cold buffer (pH 7.2) were added to each tube and centrifuged at 100000 *g* for 60 min in the case of the lysosomal and microsomal membranes and at 17000 *g* for 42 min in the case of mitochondrial outer membranes. The resulting pellets were resuspended in the medium mentioned above, and duplicate 0.1 ml aliquots were taken for protein determination and scintillation counting.

**Effects of Unlabelled Quaternary Ammonium Compounds on the Binding of $^3$H-BTTB to Lysosomal Membranes**——The lysosomal membranes preparation contained 2 mg of protein per ml; a 1 ml aliquot was mixed with an equal vol. of $^3$H-BTTB ($10^{-4}$ M, 19 μCi/ml) in buffered sucrose (pH 7.2), and various unlabelled quaternary ammonium compounds (2 ml) ranging in concentration from 0.01 to 1.0 mM were added. As the control, only sucrose was used. After incubation for 30 min at 25 °C, the suspensions were centrifuged for 60 min at 100000 *g* and the binding of $^3$H-BTTB was measured in an aliquot of the pellet, as described below.

**General Procedures**——Acid phosphatase (EC 3.1.3.2) activity was determined according to the method of Appelmans *et al.*[10] Glucose-6-phosphatase (EC 3.1.3.9) activity was determined according to the method of Swanson.[11] The liberated inorganic phosphates by these enzymatic reactions were measured by the method of Lindberg and Ernster.[12] Monoamine oxidase (EC 1.4.3.4) was determined according to the method of Turski *et al.*[13] Protein content was determined by the method of Lowry *et al.*[14] with bovine serum albumin as the standard. Radioactivity was measured by an Aloka scintillation counter, model LSC-502. The scintillation medium used consisted of 100 g of naphthalene, 4 g of 2,5-diphenyloxazole (PPO) and 0.4 g of 1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]-benzene (dimethyl POPOP) per liter of a mixture of 750 ml of dioxane, 150 ml of toluene and 100 ml of methylcellosolve. The sample (0.1 ml) was added to 10 ml of the scintillator and radioactivity was measured as described above.

## Results and Discussion

### Time Course of Binding

The time course of the binding of radioactivity to the lysosomal membranes is shown in

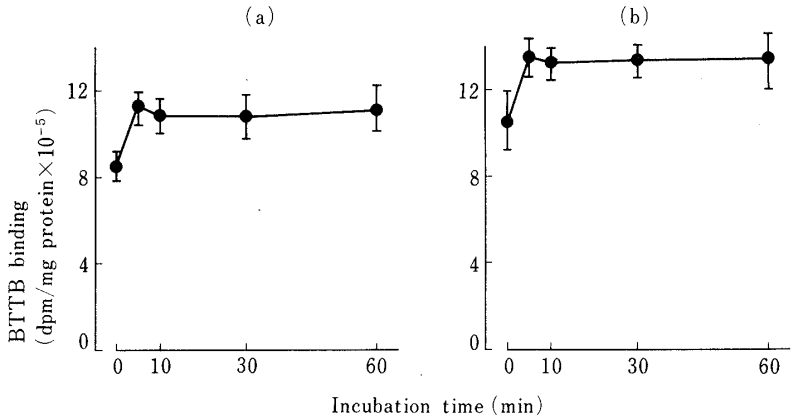


Fig. 1. Time Course of Binding of $^3$H-BTTB to Lysosomal Membranes of Rat Liver

Lysosomal membranes from rat liver were incubated with $^3$H-BTTB at 0°C (a) or 25°C (b) for different periods of time. After each period, incubation was terminated by the addition of $10^{-3}$ M unlabelled BTTB. Binding to the lysosomal membranes was also estimated. Each point is the mean ± S.D. of three experiments.

TABLE I. Specific Activities of Marker Enzymes in Rat Liver Homogenates and Other Subcellular Membrane Fractions

| | Acid phosphatase | Monoamine oxidase | Glucose-6-phosphatase |
|---|---|---|---|
| A Homogenate | 0.0121 ± 0.002 | 3.3 ± 0.2 | 0.030 ± 0.015 |
| B Lysosomal | 0.235 ± 0.022 | 2.6 ± 0.3 | 0.034 ± 0.005 |
| | | (B/C = 0.047) | (B/D = 0.12) |
| C Outer mitochondrial | 0.038 ± 0.022 | 55.9 ± 3.3 | |
| | (C/B = 0.16) | | |
| D Microsomal | 0.016 ± 0.006 | 0.9 ± 0.2 | 0.28 ± 0.06 |
| | (D/B = 0.07) | (D/C = 0.016) | |
| *R.S.A.* | B/A = 19.4 | C/A = 16.9 | D/A = 9.3 |

Values are given as means ± standard deviation.
All enzyme activities are expressed in units per mg protein per min.
*R.S.A.* = relative specific activity (specific activity found in the fraction/specific activity measured in the homogenate).

Fig. 1.

When the lysosomal membranes were incubated with $^3$H-BTTB at 0 °C or 25 °C at the indicated time intervals, the binding of radioactivity to the lysosomal membranes reached a maximum in 5 min. The binding peak of $^3$H-BTTB to the outer mitochondrial membranes was also attained 5 min after the start of incubation (data not shown). Furthermore, the peak for the microsomal membranes was also reached in 5 min, but the specific readioactivity of the microsomal membranes was much lower than that of the lysosomal membranes.

A comparison of Fig. 1a with Fig. 1b shows that the time courses of binding of radioactivity to the lysosomal membranes are similar to each other at the two temperatures. The time courses for the outer mitochondrial and microsomal membranes were similar to that of the lysosomal membranes. Thus, the binding of $^3$H-BTTB to the lysosomal membranes did not appear to be significantly temperature-dependent. This means that the binding of $^3$H-BTTB to the lysosomal membranes does not involve an enzymatic reaction. From these results, the experimental conditions for the binding of $^3$H-BTTB to lysosomal membranes were set at 30 min incubation time at 25 °C.

**Purity of the Lysosomal, Outer Mitochondrial and Microsoaml Membranes**

The purity of each subcellular membrane fraction was examined by the determination of marker enzymes. Table I records the specific activities of various enzymatic activities found in the different subcellular membrane fractions.

The relative specific activity of acid phosphatase (EC 3.1.3.2), a marker enzyme of lysosomes, with respect to the homogenate showed a 19.4-fold increase. Acid phosphatase also showed a relative specific activity of 0.16 in the outer mitochondrial membranes, representing 16% contamination with the lysosomal membranes, while it showed a relative specific activity of 0.07 in the microsomal membranes, indicating 7% contamination with the lysosomal membranes. The relative activity of monoamine oxidase (EC 1.4.3.4) in the outer mitochondrial membrane and that of glucose-6-phosphatase (EC 3.1.3.9) in the microsomal membrane showed a 16.9-fold increase and 9.3-fold increase, respectively, compared with the homogenate. The sum of the contamination of the lysosomal and microsomal membranes with the outer mitochondrial membranes was 6% based on the relative specific activities.

On the other hand, the microsomal enzyme, glucose-6-phosphatase, was not found in the outer mitochondrial membranes. Contamination of the lysosomal and outer mitochondrial membranes with the microsomal membranes was 12% based on a similar calculation. It is considered that these subcellular membranes can be used for studying the binding of BTTB to subcellular membranes.

**Binding of BTTB to Subcellular Membranes of Rat Liver**

Figure 2 illustrates some of the Lineweaver–Burk plots for the binding of $^3$H-BTTB to lysosomal (Fig. 2a), outer mitochondrial (Fig. 2b), and microsomal membranes (Fig. 2c) prepared from rat livers.

The Lineweaver–Burk plots were obtained by the addition of $^3$H-BTTB to the subcellular membranes in both the presence and absence of *N*-methylatropinium bromide (10.7 mM). Since *N*-methylatropinium bromide is very similar in structure and metabolism to BTTB,[15] it is considered that this compound antagonizes the binding of BTTB to the lysosomal

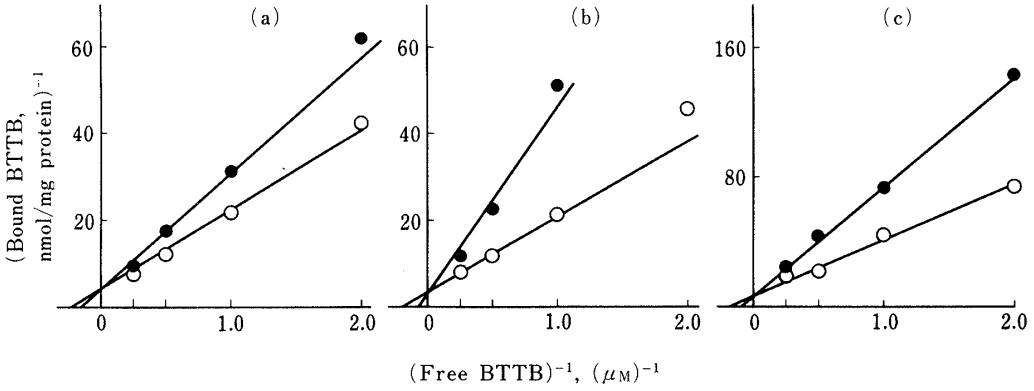


Fig. 2. Lineweaver–Burk Plots of the Binding of $^3$H-BTTB to Subcellular Membranes of Rat Liver

$^3$H-BTTB was used in the concentration range from 0.015—0.12 mM. ○ and ● indicate no addition and 10.7 mM *N*-methylatropinium bromide addition, respectively. (a) lysosomal, (b) outer mitochondrial, (c) microsomal membranes. The values of $K_m$ ($\mu$M) and $V_{max}$ (nmol BTTB per mg protein) thus obtained are as follows:

| | No addition | | Addition | |
|---|---|---|---|---|
| | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ |
| (a) Lysosomal membr. | 4.3 | 0.25 | 6.0 | 0.25 |
| (b) Outer mitochondrial membr. | 5.1 | 0.29 | 14.3 | 0.29 |
| (c) Microsomal membr. | 6.0 | 0.15 | 10.0 | 0.15 |

membranes. Thus, the effect of *N*-methylatropinium bromide on the binding of BTTB to subcellular membranes was examined.

For all membranes, the maximum binding of $^3$H-BTTB was not affected by the addition of *N*-methylatropinium bromide (10.7 mM). The values of $V_{max}$ and $K_m$ indicated that lysosomal membranes have high affinity for $^3$H-BTTB relative to other membranes.

When the lysosomal membranes were incubated in the presence of *N*-methylatropinium bromide, the Lineweaver–Burk plots showed a steeper slope than that when no addition was made. Thus, the addition of this compound to the reaction mixture causes a significant increase in the magnitude of $K_m$ but does not affect the value of $V_{max}$. *N*-Methylatropinium bromide inhibits the binding of $^3$H-BTTB by rat liver lysosomal membranes, and the inhibition seems to be of competitive type, presumably arising from the structural similarity between this compound and BTTB.

**Effect of Quaternary Ammonium Compounds on the Binding of $^3$H-BTTB to Lysosomal Membranes *in Vitro***

The effects of unlabelled quaternary ammonium compounds on the binding of $^3$H-BTTB to lysosomal membranes were studied *in vitro* to elucidate the mode of the binding of $^3$H-BTTB to rat liver lysosomal membranes. The results are shown in Tables II, III, and IV.

As shown in Table II, methacholine and carbachol had no inhibitory effect on $^3$H-BTTB binding. The binding was significantly decreased by the addition of choline and acetylcholine at a final concentration of 1 mM. However, it is considered that acetylcholine readily degrades to choline at neutral pH, so the action found with acetylcholine seems to be the same as that of choline. At present the action of choline cannot be adequately explained. No significant inhibitory effect was observed with betaine over a range of concentration from 0.01 to 1.0 mM. CTAB is an aliphatic and long chain quaternary ammonium compound having a kind of detergent action, and it caused a significant decrease at final concentrations of 0.1 and 1 mM. Rhodopsin-like protein from the purple membranes is known to be soluble in CTAB.[16] Accordingly, it is considered that the binding capacity decreases because of the solubilization of the lysosomal membranes by this compound.

Ethidium bromide, having a phenanthridinium structure is known[17] to be excreted

Table II. Effects of Monoquaternary Ammonium Compounds on the Binding of $^3$H-BTTB to Lysosomal Membranes of Rat Liver

| Compounds | Extent of binding, % difference | | |
|---|---|---|---|
| | 0.01 mM | 0.1 mM | 1.0 mM |
| Choline | +0.7 | −5.6 | −8.5[a] |
| Acetylcholine | +6.3 | +2.7 | −10.1[a] |
| Methacholine | +3.0 | +1.4 | +0.3 |
| Carbachol | +0.6 | +1.8 | +0.9 |
| Betaine | +4.6 | +1.2 | −3.0 |
| Ethidium bromide | −3.7 | −5.2 | −26.3[a] |
| CTAB | −0.7 | −33.9[a] | −35.9[a] |
| Anisotropine | +3.2 | −0.2 | −12.4[a] |

Freshly prepared lysosomal membranes from rat liver were mixed with 0.1 mM $^3$H-BTTB (19 $\mu$Ci/ml). To this mixture, various concentrations of the compounds indicated were added and incubated for 30 min at 25 °C. After incubation, the suspensions were centrifuged for 60 min at 100000 ***g*** and the radioactivity of $^3$H-BTTB was measured in the pellet fraction. Each value is the mean of three samples. The values show the difference in the percentages when the compounds were present and absent.

CTAB: Cetyl trimethyl ammonium bromide. Cetrimonium bromide.

*a*) $p<0.01$.

TABLE III. Effects of Bis- and Triquaternary Ammonium Compounds on the Binding of $^3$H-BTTB to Lysosomal Membranes of Rat Liver

| Compounds | Extent of binding, % change | | |
|---|---|---|---|
| | 0.01 mM | 0.1 mM | 1.0 mM |
| Hexamethonium | +3.0 | −0.6 | −7.6[a] |
| Succinylcholine | +6.1 | +2.8 | −6.2[a] |
| Decamethonium | +2.2 | −1.7 | +0.5 |
| Indocyanine green | +9.9 | −8.9[a] | −24.7[a] |
| *d*-Tubocurarine | +0.1 | +6.6 | −4.2 |
| Gallamine | +9.4 | +2.9 | +5.2 |

The experimental procedure is described in the legend to Table I.
*a*) $p<0.01$.

rapidly into the bile from liver, like BTTB. Anisotropine is a quaternary ammonium compound with a tropane skeleton, and is very similar to BTTB in both structure and pharmacological action. The binding of BTTB was also decreased significantly by the addition of these drugs at a final concentration of 1 mM. In addition, since the drug mentioned above are lysosomotropic drugs, they may compete with each other in inhibiting the binding of BTTB.

The effects of bis- and tri-quaternary ammonium compounds on the binding of $^3$H-BTTB to lysosomal membranes were also examined. The results are shown in Table III.

Indocyanine green was inhibitory at a final concentration of 1 mM. This dye is known to be excreted into the bile without conjugation after being taken up by the liver.[18] Meijer *et al.*[19] recently reported that subcellular fractionation of the liver 15 min after the intravenous administration of *d*-tubocurarine confirmed the localization of this drug in the lysosomes.

It is also known that *d*-tubocurarine is actively secreted into bile. In order to investigate whether or not a competitive phenomenon is present, the binding of $^3$H-BTTB to lysosomal membranes was examined in the presence of *d*-tubocurarine *in vitro*. To investigate whether or not the number of quaternary ammonium groups affects the binding of $^3$H-BTTB to lysosomal membranes, the effect of gallamine, a triquaternary ammonium compound, on the binding of $^3$H-BTTB to lysosomal membranes was also examined. The results showed that these reagents exerted no inhibitory effect on BTTB binding.

The effects of polymethylene–bisquaternary ammonium compounds such as hexamethonium ($C_6$), succinylcholine ($C_8$), and decamethonium ($C_{10}$) on BTTB binding are also shown in Table III. It has been shown[20] by whole-body autoradiography that decamethonium accumulates in the liver. To determine whether or not the distance between two quaternary ammonium groups in a competing compound affects the binding of $^3$H-BTTB to lysosomal membranes, the effects of hexamethonium, succinylcholine and decamethonium were examined. It was found that binding was significantly decreased by the addition of hexamethonium and succinylcholine, each at a final concentration of 1 mM. For such competition, with BTTB binding it may be necessary that these drugs have chain lengths of up to eight carbon atoms and that the distance between the two quaternary ammonium groups does not exceed 14 Å.

Table IV shows the effects of cholinesterase inhibitors and tertiary amines on the binding of $^3$H-BTTB to lysosomal membranes *in vitro*.

As is evident from Table IV, neither of the cholinesterase inhibitors (*i.e.*, paraquat and neostigmine) showed detectable effects on $^3$H-BTTB binding. From the results in Table IV, a 30-min incubation period in the presence of 1 mM quinacrine or 1 mM chloroquine, both having

TABLE IV. Effects of Cholinesterase Inhibitors and Tertiary Amines on the Binding of $^3$H-BTTB to Lysosomal Membranes of Rat Liver

| Compounds | Extent of binding, % change | | |
|---|---|---|---|
| | 0.01 mM | 0.1 mM | 1.0 mM |
| Neostigmine | +1.4 | −1.8 | +3.7 |
| Paraquat | −3.7 | +2.8 | +5.1 |
| Atropine | −3.0 | −4.2 | −4.6 |
| Chloroquine | +8.8 | −1.9 | −27.0[a] |
| Quinacrine | +3.1 | +12.2 | −28.9[a] |

The experimental procedure is described in the legend to Table I.
*a*) $p<0.01$.

high affinity toward lysosomes, significantly reduces the binding reaction. These compounds, like BTTB, have weakly basic properties and are lysosomotropic drugs,[21] and thus it is considered that they act competitively to inhibit the binding of BTTB to lysosomal membranes. Further, atropine is a tertiary amine having a tropane skeleton. Table IV indicates that the binding tends to decrease with increase in the concentration of this drug.

The present binding activity studies were carried out at pH 7.2. Further investigation is necessary to elucidate the pH-dependency of the binding of BTTB to lysosomal membranes and of the competitive inhibition caused by basic compounds.

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