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# Partial Characterization of Specific Cantharidin Binding Sites in Mouse Tissues

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**SUMMARY**

The mode of action of cantharidin, the natural vesicant of blister beetles, is examined by radioligand binding studies with mouse tissues. [$^3$H]Cantharidin undergoes specific and saturable binding with the liver cytosol, which is characterized as follows: $K_d$ and $B_{max}$ values of 30 nM and 1.8 pmol/mg of protein, respectively; linearity with respect to protein concentration; pH optimum of 6.5 to 7.5; association and dissociation half-times of 20 min and 12 hr, respectively; and 50% inhibition by $Mg^{2+}$ at 70 $\mu$M, $Ca^{2+}$ at 224 $\mu$M, pyrophosphate at 27 $\mu$M, and nucleotide triphosphates at 52–81 $\mu$M. The binding site undergoes a loss of activity at 45° or higher. The toxicological relevance of this specific [$^3$H]cantharidin binding site of mouse liver cytosol is established in three ways. First, the potency of 15 active cantharidin analogs for inhibiting [$^3$H]cantharidin binding is correlated with their acute toxicity to mice ($r = 0.829$). Second, 26 related compounds that are inactive in inhibiting [$^3$H]cantharidin binding are also of little or no toxicity to mice. Finally, the binding of [$^3$H]cantharidin to liver cytosol from mice poisoned with increasing amounts of unlabeled cantharidin is inhibited in a dose-dependent manner. [$^3$H]Cantharidin also specifically binds to cytosol fractions of blood, brain, heart, kidney, lung, pancreas, skin, spleen, and stomach. The characteristics of the specific binding site in brain are very similar to those determined in liver with respect to $K_d$, $B_{max}$, association/dissociation kinetics, and sensitivity to inhibitors. It therefore appears that the toxicity of cantharidin and related oxabicycloheptanes, including the herbicide endothal, is attributable to binding at a specific site in liver and possibly other tissues.

Cantharidin (*exo*, *exo*-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride), a natural toxicant of blister beetles (1, 2), has been the cause of numerous cases of human poisonings due to its purported aphrodisiac properties (3, 4). Livestock have also been poisoned by consuming feed contaminated with blister beetles (5, 6). It is estimated that less than 65 mg of cantharidin is lethal to humans (3). In horses, ingestion of 4 to 6 grams of whole blister beetles or oral doses of purified cantharidin as low as 0.5 mg/kg can be fatal (6, 7). The IP $LD_{50}$ of cantharidin in mice is approximately 1 mg/kg (8).

Ingestion of cantharidin or whole blister beetles leads to severe irritation and ulceration of the epithelial linings of the gastrointestinal and urinary tracts (3–7). Dermal contact leads to blister formation and separation of cells known as acantholysis (9, 10). Cantharidin can also cause severe congestion and edema of the liver, particularly after IP administration (6, 11–13). Histologically, the congestion and edema of liver tissue is very similar to cantharidin-induced acantholysis of skin (9, 10, 12). For this reason, the liver is often used as a model target organ for studying the biochemical effects of cantharidin on mammalian tissues (12–15). Earlier studies attributed cantharidin's mode of action to interference with mitochondrial respiration (14, 15) or to activation of an acantholytic factor (9, 10, 16).

We recently synthesized [$^3$H]cantharidin and identified a specific binding site in mouse liver cytosol (17). No appreciable binding occurred with any of the liver membrane fractions. Structure-activity assays with 22 oxabicycloheptane-dicarboxylic acid analogs, including the widely used herbicide endothal (18), suggest that this cantharidin-binding site interaction either represents, or serves as a model for, the mechanism of toxic action (17). The objectives of the present study were to more fully characterize the interaction of [$^3$H]cantharidin with its binding site in mouse liver cytosol, to further evaluate the toxicological relevance of this interaction by additional structure-activity assays and by assaying for *in vivo* inhibition of the binding site, and, finally, to determine whether similar binding sites occur in other tissues.

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**ABBREVIATION:** IP, intraperitoneal.

## Experimental Procedures

**Materials.** [$^3$H]Cantharidin (14 Ci/mmol, >98% radiochemical purity) was prepared by a procedure outlined in our earlier report (17). The dehydrothiocantharidin precursor (19) (2.5 mg) was dissolved in ethyl acetate (3 ml) and 10% palladium on carbon catalyst (2.4 mg) was added. After freeze-thaw degassing, 1 atmosphere of 100% tritium gas was admitted and the reaction was stirred 4 hr at 25°. Volatile tritium was removed by partial evaporation of the solvent and addition of fresh ethyl acetate three times. The catalyst was filtered off and the filtrate was evaporated and redissolved in ethanol (50 ml). An aliquot (0.7 ml) of this stock solution was added to ethanol (2 ml) containing Raney nickel slurry (20) (2–3 mg). After stirring at reflux for 3 hr under nitrogen, the solution was filtered and the [$^3$H]cantharidin was purified by high pressure liquid chromatography followed by confirmation of its identity and determination of its specific activity as previously described (17). The radiochemical purity was determined by thin layer chromatography on silica gel 60 F-245 chromatoplates developed with chloroform to give an $R_F$ for cantharidin of 0.42.

Unlabeled cantharidin was obtained from K & K Laboratories (Plainview, NY). Cantharidic acid (as the disodium salt) was prepared by hydrolysis of cantharidin with 1.0 N NaOH (13). Endothal monohydrate (>99% purity, Lot RLW 7-74) was from Pennwalt Corporation (Tacoma, WA). Other cantharidin analogs were from syntheses reported earlier (8). All biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

**Preparation of tissue cytosol.** Fresh tissues from male Swiss-Webster mice were homogenized at 20% w/v in ice-cold 20 mM Tris-maleate pH 7.4 buffer and centrifuged at 15,000 × $g$ for 15 min at 0–4°. The resulting supernatant was centrifuged at 105,000 × $g$ for 60 min. The cytosol (supernatant at 105,000 × $g$) was dialyzed four consecutive times (12 hr each) against 50 volumes of ice-cold buffer and aliquots were frozen and stored at −70°. Protein was determined by the method of Bradford (21).

**Binding assays.** The standard incubation mixture for binding assays consisted of 5 nM [$^3$H]cantharidin and 0.5 mg of cytosolic protein in 1 ml of 20 mM Tris-maleate pH 7.4 buffer. Incubations were carried out with slow shaking at 37° for 90 min unless indicated otherwise. After incubation all samples were simultaneously filtered under vacuum through Whatman GF/C glass fiber filters and rapidly rinsed with buffer (2 × 5 ml) using a Brandel Cell Harvester (Model M-24R; Brandel Instruments, Gaithersburg, MD). The filters were presoaked for at least 1 hr in 0.3% polyethylenimine (22). Radioactivity retained on the filters was quantitated by liquid scintillation counting.

Unlabeled cantharidic acid (10 μM final concentration) was added to determine nonspecific binding. Specific binding is defined as the difference between total binding (with [$^3$H]cantharidin only) and nonspecific binding. Cantharidin and cantharidic acid are equipotent in inhibiting [$^3$H]cantharidin binding. The apparent dissociation constant ($K_d$) and the maximum number of binding sites ($B_{max}$) were determined by linear regression analysis of Scatchard plots.

**Association/dissociation kinetics.** The rate of association of [$^3$H]cantharidin with its binding site was determined by filtration of the incubation mixture at various time intervals ranging from 15 min to 3 hr. Dissociation was determined after first equilibrating [$^3$H]cantharidin with the cytosol for 90 min at 37°. Unlabeled cantharidic acid was then added and specific binding was measured at various times up to 24 hr. The observed association rate constant ($k_{obs}$) and the dissociation rate constant ($k_{-1}$), which were used to calculate the half-time values ($t_{½}$), were determined from natural logarithmic transformations of the association and dissociation binding data, respectively. The pseudo-first order association rate constant ($k_{+1}$) was calculated from $k_{-1}$ and $K_{obs}$ (23).

**Inhibition assays.** Analogs or endogenous compounds were added as solutions in acetone (10 μl) or buffer (10 to 100 μl) to the incubation mixtures and assayed under the standard conditions. The $IC_{50}$ values (concentrations for 50% inhibition) were determined by linear regression analysis.

***In vivo* binding.** Male Swiss-Webster mice weighing 20–23 g were administered cantharidin at 0, 1, 3, and 10 mg/kg by IP injection in dimethyl sulfoxide/methoxytriglycol (1:1) (50 μl). Thirty min after treatment, the mice were sacrificed and the liver cytosol was prepared as described above. To remove unbound cantharidin and low molecular weight endogenous compounds, 0.5 mg of cytosolic protein from each mouse was applied to a Sephadex G25-150 column (1.0 × 12 cm) and eluted with 5 ml of assay buffer. One-ml fractions were collected and assayed for specific binding under the standard conditions. One sample from each animal was assayed for total binding and another for nonspecific binding. The column was regenerated by washing with 15 ml of buffer.

**Toxicity assays.** $LD_{50}$ values (median lethal doses) were determined 72 hr after IP administration of the compounds to male Swiss-Webster mice (8).

## Results

**Characteristics of liver [$^3$H]cantharidin binding site.** [$^3$H]Cantharidin interacts in a specific and saturable manner with a binding site in mouse liver cytosol with apparent $K_d$ and $B_{max}$ values of 30 nM and 1.8 pmol/mg protein, respectively (17). Specific binding is linear with respect to protein concentration (Fig. 1) and is optimal within the pH range of 6.5 to 7.5 (Fig. 2). The binding site undergoes inactivation on 10-min exposure to temperatures of 45° or higher (Table 1). The [$^3$H]cantharidin-binding site complex is completely dissociated within 1 min on addition of 1 ml of 20% trichloroacetic acid to the incubation mixture, demonstrating that covalent binding is not involved.

**Association and dissociation kinetics of specific [$^3$H]cantharidin binding to liver cytosol.** [$^3$H]Cantharidin associates with its binding site under the standard assay conditions with a $t_{½}$ of 19.5 min and a $k_{obs}$ of 0.0356 $min^{-1}$ (Fig. 3A). Equilibrium, within normal experimental variation, is achieved at 90 min. In contrast, the rate of dissociation of [$^3$H]cantharidin from its binding site is quite slow with a $t_{½}$ of 11.5 hr and a $k_{-1}$ of 0.060 $hr^{-1}$ (Fig. 3B). Using $k_{-1}$ and a calculated $k_{+1}$ of $2.26 \times 10^5$ $min^{-1}$ $M^{-1}$, the $k_d$ obtained from the kinetic data is 0.15 nM. This is 200 times less than the $k_d$ derived from the saturation experiments (17).

**Effects of endogenous substances on [$^3$H]cantharidin binding to liver cytosol.** $Mg^{2+}$ and $Ca^{2+}$ are fairly potent inhibitors with $IC_{50}$ values of 70 and 224 μM, respectively (Table

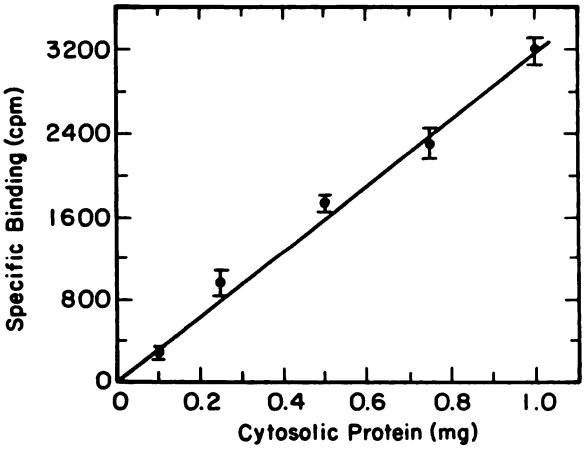


**Fig. 1.** Relation of specific [$^3$H]cantharidin binding to amount of mouse liver cytosolic protein. Assays were performed under the standard conditions using 5 nM [$^3$H]cantharidin. Each *point* represents the mean and standard error of three experiments.

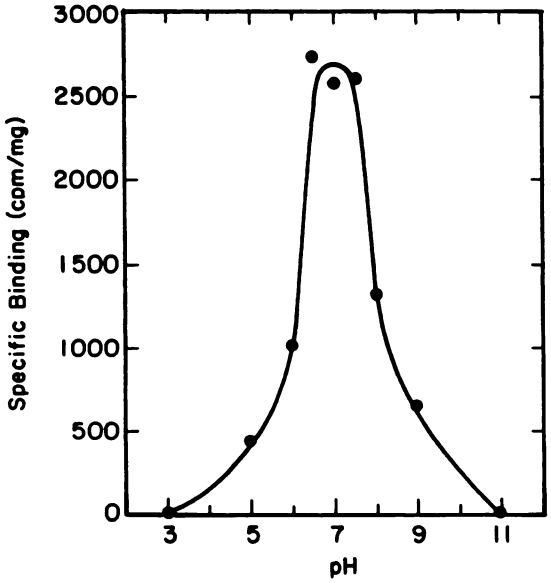


**Fig. 2.** Effect of pH on specific [$^3$H]cantharidin binding to mouse liver cytosol. Assays were conducted under the standard conditions using 5 nM [$^3$H]cantharidin and 0.5 mg of cytosolic protein. Each *point* is the average of two experiments.

TABLE 1

**Thermal stability of the mouse liver [$^3$H]cantharidin binding site**

| Preincubation[a] | Specific binding[b] |
|---|---|
| °C | % of control |
| 37 | 100 |
| 45 | 83 |
| 55 | 36 |
| 65 | 0 |

[a] The cytosol (0.5 mg of protein) was preincubated for 10 min at the indicated temperature, then [$^3$H]cantharidin was added and binding was determined under the standard conditions (37°, 90 min).

[b] Average of two experiments.

2). $Na^+$, $K^+$, and inorganic phosphate are not active. Pyrophosphate ($PP_i$) and the nucleotide triphosphates (ATP, GTP, and UTP) are potent inhibitors with $IC_{50}$ values ranging from 27 to 81 μM. Although not tabulated, the synthetic ATP analog β,γ-methyleneadenosine 5′-triphosphate with a nonhydrolyzable terminal phosphate group is also a fairly good inhibitor with an $IC_{50}$ of 190 μM. The nucleotide diphosphates ADP and GDP are less effective with $IC_{50}$ values of 170 and 240 μM, respectively. Other phosphate-containing compounds examined and glutathione are less active or inactive with $IC_{50}$ values ≥ 771 μM. The inhibitory actions of $PP_i$ and ATP are blocked by $Mg^{2+}$ at molar ratios of 1:3 for $PP_i$ and 1:1 for ATP. Several exogenous compounds including phenylmethanesulfonyl fluoride, *N*-ethylmaleimide, and EDTA are also ineffective as inhibitors ($IC_{50}$ values > 1,000 μM).

**Comparative potencies of 41 oxabicycloheptane-carboxylic acid derivatives as inhibitors of [$^3$H]cantharidin binding to liver cytosol and as acute toxicants to mice.** There is a good correlation ($r$ = 0.829) for the 15 active compounds between potency in inhibiting [$^3$H]cantharidin binding and toxicity to mice (Fig. 4). These include dicarboxylic acids, anhydrides, esters, and an anilide. Endothal is of intermediate activity both as an inhibitor and as a toxicant. Dimethyl substitution at the 2,3-positions to yield cantharidic acid (and the corresponding anhydride cantharidin) greatly increases receptor potency and acute toxicity. The 2,3-trimethylene-anhydride is also more active than endothal although the 2-bromo-derivative is less active. Monosubstitution at the 5-position weakens but does not eliminate toxicity and, except for the *endo*-5-carboxy group, also reduces receptor potency. Dehydrogenation at the 5,6-position and esterification of one or both carboxylic acid groups also reduces but does not eliminate biological activity. Formation of the 4-chloroanilide weakens receptor affinity but does not affect toxicity. Cantharidin, endothal-anhydride and 5,6-dehydroendothal-anhydride are each more toxic than the corresponding dicarboxylic acid, although their receptor potency remains almost unchanged. All other modifications of the oxabicycloheptane-dicarboxylic acid structure eliminate all activity. These include substitutions at the 1,4- and 5,6-positions, removal of one carboxylic acid group, replacement of the bridgehead oxygen with sulfur, and isomerization of the dicarboxylic acid groups to *endo,endo-* or *exo,exo-*. The inactive analogs (Fig. 4) are the *endo,exo-* and *endo,endo*-isomers of endothal, *endo,endo*-1,4-dimethylendothal, and 23 derivatives of endothal [1-methyl-; 1-ethyl-; 1,4-dimethyl-; *endo,endo*-5,6-dimethyl-; *exo*-5,6-epoxy-; *exo,exo*-5,6-dihydroxy-; *exo,exo*-5,6-methylenedioxy-; *endo,exo*-5,6-dibromo-; *endo,exo*-5,6-dichloro-; *exo,exo*-5,6-dibromo-; 5,6-phenylene-anhydride; 2,3-dimethyl-5,6-phenylene-anhydride; *endo*-2,3-dimethyl-anhydride; 2,3-dehydro-; *exo*-2-monocarboxylic

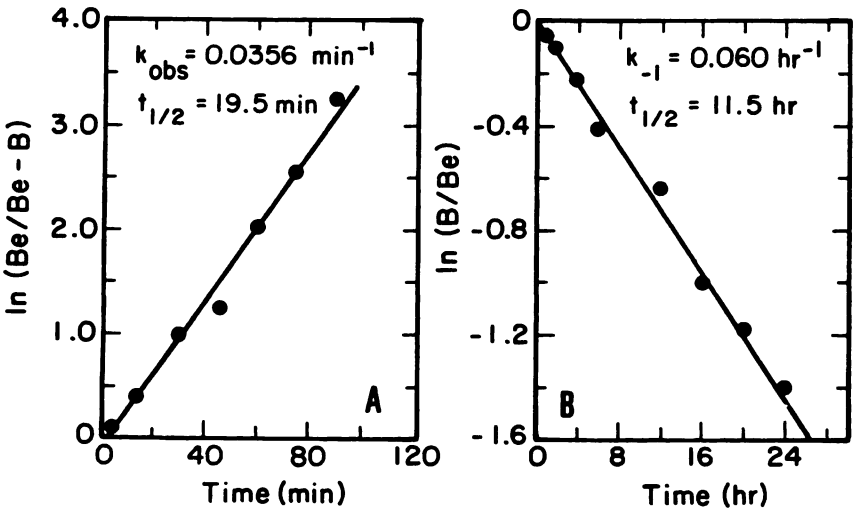


**Fig. 3.** Kinetics of association (*left panel*) and dissociation (*right panel*) of specific [$^3$H]cantharidin binding to mouse liver cytosol. Dissociation of specifically bound [$^3$H]cantharidin at equilibrium ($B_e$) was initiated by the addition of 10 μM unlabeled cantharidic acid. The amount of residual specifically bound radioligand ($B$) was then determined at the times indicated. Linear regression analysis gives correlations of 0.994 and 0.995 for the association and dissociation data, respectively. Each *point* represents the mean of three experiments.

TABLE 2

**Inhibitory potency of endogenous substances on [$^3$H]cantharidin binding to mouse liver cytosol**

| Substance[a] | $IC_{50}$[b] |
|---|---|
| | μM |
| Salts | |
| $MgCl_2$ | 70 |
| $CaCl_2$ | 224 |
| NaCl | >100,000 |
| KCl | >100,000 |
| Phosphates | |
| $PP_i$ | 27[c] |
| GTP | 52 |
| UTP | 75 |
| ATP | 81[c] |
| ADP | 170 |
| GDP | 240 |
| Coenzyme A | 771 |
| UDP | 1,000 |
| AMP | >1,000 |
| cAMP | >1,000 |
| NADP | >1,000 |
| FAD | >1,000 |
| Inorganic phosphate | >6,000 |

[a] Substances were added as buffer solutions (10 to 100 μl) to the incubation mixtures. [$^3$H]Cantharidin binding was then assayed under the standard conditions. Three or four concentrations of each substance were used giving between 5 and 95% inhibition. The $IC_{50}$ for glutathione was >1,000 μM.

[b] Average of two experiments each run in duplicate.

[c] Inhibition is blocked by $MgCl_2$ at a molar ratio of 1:3 relative to $PP_i$ or 1:1 relative to ATP.

acid; *endo*-2-monocarboxylic acid; 2,3-C(O)OCH$_2$—; 2,3-C(O)CH$_2$C(O)—; *endo,endo*-7-CH$_2$—; *exo,exo*-7-S—; 2,3-C(O)N(H)C(O)—; 2,3-C(O)N(CH$_2$CH$_3$)C(O)—; and 2,3-C(O)N(4-chlorophenyl)C(O)—].

***In vivo* inhibition of [$^3$H]cantharidin binding site.** The binding of [$^3$H]cantharidin to liver cytosol of mice pretreated for 30 min with IP administered unlabeled cantharidin is inhibited in a dose-dependent manner correlated with the poisoning signs, i.e., 26, 48, and 69% inhibition at doses of 1, 3, and 10 mg/kg, respectively (Table 3). This can be considered as *in vivo* inhibition based on preliminary experiments, which showed that (a) unbound cantharidin is effectively removed from the cytosol by chromatography on Sephadex G25-150, (b) bound cantharidin remains with the cytosolic proteins during separation, and (c) the activity of the binding site is unchanged on chromatography (data not shown). A potential artifact of inhibitor binding during sample preparation is not likely because cantharidin binding does not occur at 0–4°.

**Specific binding of [$^3$H]cantharidin to brain and other tissues.** [$^3$H]Cantharidin undergoes specific binding to the cytosolic fractions of several mouse tissues (Table 4). The amount of binding to the brain, stomach, skin, and heart is significantly greater than that to the liver under the same conditions. On the other hand, whole blood has a significantly lower capacity for binding [$^3$H]cantharidin.

The characteristics of [$^3$H]binding to liver and brain cytosol are compared in Table 5. Although the same binding affinity ($K_d$ = 29 to 30 nM) is found for both tissues, there is a greater density of binding sites in the brain cytosol, i.e., $B_{max}$ values of 3.4 pmol/mg brain protein versus 1.8 pmol/mg of liver protein. A saturation isotherm and a Scatchard plot of the specific binding of [$^3$H]cantharidin to dialyzed mouse brain cytosol are shown in Fig. 5. The Hill coefficient of 1.0 to 1.1 for each tissue indicates no cooperativity between the binding sites. There are

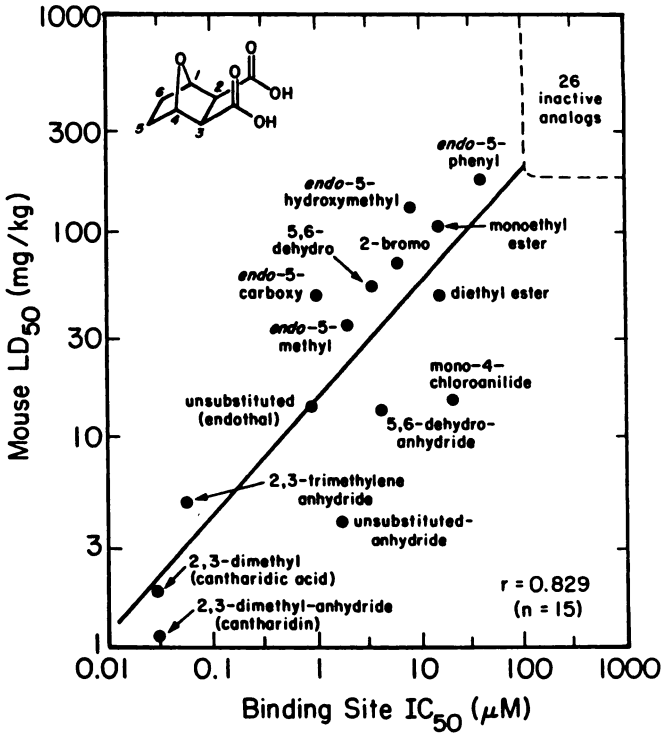


**Fig. 4.** Correlation for 41 oxabicycloheptane-carboxylic acid derivatives between potency as inhibitors of [$^3$H]cantharidin binding to dialyzed liver cytosolic protein and acute toxicity to mice. The structure shown is that of endothal, the unsubstituted dicarboxylic acid analog with the *exo,exo*-configuration. The binding assays were performed under the standard conditions. The compounds were added to the incubation mixtures as solutions (10 μl) in water or acetone. At least four concentrations of each derivative were assayed giving between 5 and 95% inhibition. $LD_{50}$ values were determined 72 hr after IP administration (8). Results represent the averages of two experiments, each run in duplicate. $IC_{50}$ and $LD_{50}$ values for the inactive compounds are >100 μM and/or >400 mg/kg. respectively.

TABLE 3

***In vivo* inhibition of [$^3$H]cantharidin binding to liver cytosol from mice 30 min after IP administration of unlabeled cantharidin**

| Cantharidin[a] | Specific binding[b] |
|---|---|
| mg/kg | % of control |
| 0 | 111 ± 16 |
| 1 | 74 ± 10[c] |
| 3 | 52 ± 9[c] |
| 10 | 31 ± 9[c,d] |

[a] The mice were treated and the liver cytosol was prepared as described in the text. The poisoning signs (see Ref. 13) were none at 1 mg/kg, moderate at 3 mg/kg, and severe at 10 mg/kg.

[b] Unbound cantharidin was removed by chromatography on Sephadex G25-150. The control value is specific binding for the cytosol of untreated mice (which was not passed through the Sephadex column). The results represent the means and standard errors for three mice at each dose.

[c] Significantly different from 0 mg/kg dose group ($p < 0.05$) by ANOVA and Duncan's multiple range test.

[d] Significantly different from 1 mg/kg dose group ($p < 0.05$) by ANOVA and Duncan's multiple range test.

differences between the liver and brain cytosol with respect to their association and dissociation rates. Whereas the liver exhibits monophasic rates of association and dissociation (Fig. 3), the brain shows a distinct biphasic response for both parameters, i.e., $\alpha$ and $\beta$ (Fig. 6), suggesting the presence of two populations of binding sites. There is an excellent agreement ($r$ = 0.998) between the liver and brain binding sites with

TABLE 4

**Specific binding of [$^3$H]cantharidin to cytosolic fractions of various mouse tissues**

| Tissue | Specific binding[a] |
|---|---|
| | % of liver |
| Brain | 364 ± 75[b] |
| Stomach | 200 ± 20[b] |
| Skin | 151 ± 20[b] |
| Heart | 135 ± 10[b] |
| Kidney | 131 ± 28 |
| Spleen | 122 ± 10 |
| Lung | 90 ± 10 |
| Whole blood | 36 ± 14[b] |

[a] Specific binding was determined under the standard conditions. The results represent the means and standard errors for three mice.
[b] Significantly different from liver ($p < 0.05$) by Student's $t$ test.

TABLE 5

**Characteristics of the specific [$^3$H]cantharidin binding sites of mouse liver and brain cytosol**

| Binding parameters | Liver[a] | Brain[a] |
|---|---|---|
| $K_d$ (nM) | 30 | 29 |
| $B_{max}$ (pmol/mg of protein) | 1.8 | 3.4 |
| Hill coefficient[b] | 1.0 | 1.1 |
| Association $t_{1/2}$ (min) | 19.5 | 12, 48[c] |
| Dissociation $t_{1/2}$ (hr) | 11.5 | 2.3, 17.5[c] |
| $IC_{50}$ of inhibitors[d] | Liver | Brain |
| | μM | |
| 2,3-Dimethyl- (cantharidic acid) | 0.029 | 0.044 |
| 2,3-Trimethylene-anhydride | 0.053 | 0.098 |
| Unsubstituted (endothal) | 0.93 | 1.3 |
| *endo*-5-Methyl- | 2.0 | 2.0 |
| 2-Bromo- | 6.6 | 6.3 |
| 1,4-Dimethyl- | >100 | >100 |
| 5,6-Dimethyl- | >100 | >100 |
| *endo*, *exo*-Endothal | >100 | >100 |

[a] Parameters were determined as described in the text. The results represent the averages of at least two experiments each run in duplicate.
[b] Determined by linear regression analysis of the binding data from a Hill plot.
[c] The biphasic association and dissociation rates were determined as indicated in Fig. 6.
[d] See Fig. 4 for generic structure.

respect to their affinity for five active oxabicycloheptane-dicarboxylic acid and anhydride derivatives (Table 5).

## Discussion

The acute toxicity of cantharidin and other oxabicycloheptane-dicarboxylic acid derivatives is directly related to their interaction with a binding site in the cytosolic fraction of liver and possibly other tissues. The correlation between binding affinity and acute toxicity is excellent for the active dicarboxylic acids ($r = 0.962$, $n = 8$; Ref. 17) and is still very good when all other active analogs are included ($r = 0.829$, $n = 15$). This relationship is further strengthened by the finding that all analogs that are not toxic to mice ($LD_{50} \geq 400$ mg/kg) are also not active as inhibitors ($IC_{50}$ values $\geq 100$ μM). Additionally, the binding of [$^3$H]cantharidin to liver cytosol from mice poisoned with increasing amounts of unlabeled cantharidin is inhibited in a dose-dependent manner.

[$^3$H]Cantharidin binding may alter the binding site conformation in a manner to prevent bound cantharidin from being released. This conclusion is based on the 200-fold greater apparent affinity derived from saturation experiments than when calculated from kinetic data, a discrepancy that undoubtedly arises from the extremely slow rate of dissociation because it is not due to covalent derivatization. On a similar basis, ryanodine appears to be occluded on interaction with its $Ca^{2+}$-dependent receptor complex (24).

The topography of the cantharidin binding site is defined in part by the structural requirements of the oxabicycloheptane-dicarboxylic acids for receptor inhibition. The dicarboxylic acid groups (or their corresponding anhydride) must be unaltered and in the *exo,exo*-position for optimal orientation with respect to the bridgehead oxygen. This orientation is improved by suitable substituents in the 2- and 3-positions. Monocarboxylic acids, isomers with *endo,exo-* or *endo,endo*-dicarboxylic acid groups, and an analog in which the bridgehead oxygen is replaced with sulfur are all inactive, further indicating the importance of this oxygen "triad". An unsubstituted oxabicycloheptane ring at carbons 1, 4, 5, and 6 is required for optimal binding and, consequently, the fewer the modifications at these sites, the greater the activity. Thus, 1,4- or 5,6-substitution completely eliminates all activity regardless of the isomeric positions of the dicarboxylic acid groups. Monosubstitution at carbon 5 and 5,6-dehydrogenation reduce, but do not eliminate, biological activity. Interestingly, these overall structural requirements are similar to those observed for herbicidal activity (8), indicating that a similar binding site may exist in susceptible plants. For mammals, the anhydrides are somewhat more toxic than the corresponding dicarboxylic acids although receptor potency is not very different, suggesting more efficient transport of the lipophilic anhydrides to the target site.

The endothal anhydride analog with the —C(O)OC(O)— substituent replaced by —C(O)SC(O)— is the most toxic compound of this type prepared to data (8) yet its inhibitory potency ($IC_{50}$, 1.4 μM) is less than anticipated from its $LD_{50}$ (0.3 mg/kg). This apparent discrepancy indicates that it may differ from the other analogs in its distribution, metabolism, or site of action.

The cantharidin binding site interacts with some metals and phosphorus-containing compounds but there is no direct evidence that the binding site is a metallo- or phosphoprotein. The chelating properties of cantharidin (8, 25) might be important if the binding site contains a divalent cation but chelation of free divalent cations could lead to deactivation of cantharidin. $Mg^{2+}$, ADP, and ATP inhibit [$^3$H]cantharidin binding *in vitro* when tested at levels normally found in liver (26, 27), yet [$^3$H]cantharidin binds readily with nondialyzed cytosol and *in vivo*. Based on the inhibitory effects of $PP_i$ and the nucleotides, it is possible that the [$^3$H]cantharidin binding site is an enzyme that in some way utilizes or metabolizes nucleotides and/or $PP_i$. Additional research is clearly necessary to further delineate these unusual interactions.

[$^3$H]Cantharidin undergoes specific binding not only in liver but also in every other tissue assayed, consistent with the toxicity of both cantharidin (3–7, 9–13) and endothal (28) to many organs. The similarity in characteristics of the brain and liver binding sites may extend to other tissues as well. There are no reports that cantharidin or endothal is neurotoxic. Perhaps the blood/brain barrier prevents accumulation of toxic levels in the brain, a possibility consistent with the distribution of [$^{14}$C]endothal in rats (29). Nevertheless, the brain is a potential organ for isolating the cantharidin binding site due to its relatively high $B_{max}$ value.

The toxic action of cantharidin is proposed to involve either

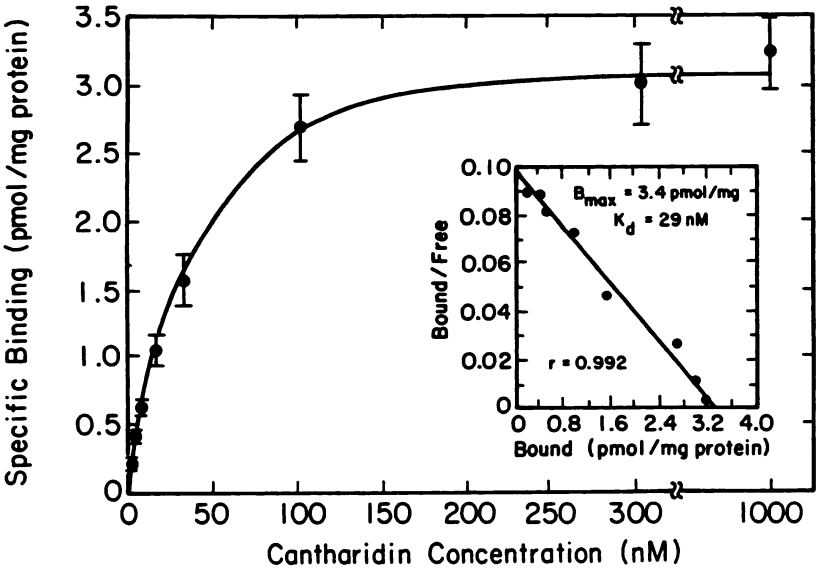


Fig. 5. Specific binding of [$^3$H]cantharidin to mouse brain cytosol shown as a saturation isotherm and a Scatchard plot (*insert*). Assays were conducted under the standard conditions. Each *point* represents the mean and standard error of three experiments.

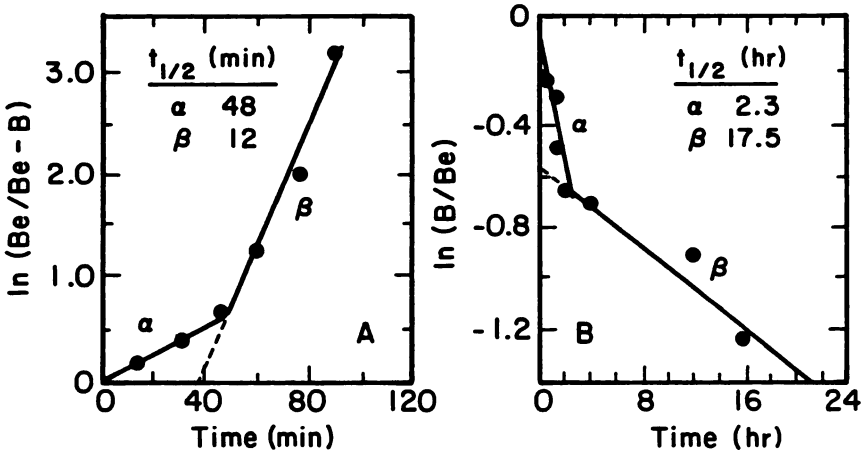


Fig. 6. Kinetics of association (*left panel*) and dissociation (*right panel*) of specific [$^3$H]cantharidin binding to mouse brain cytosol. Assays were performed as described in Fig. 3. Half-time values for components $\alpha$ and $\beta$ were determined by linear regression analysis. Each *point* represents the mean of three experiments.

inhibition of mitochondrial respiration (14, 15) or activation of an acantholytic factor in the soluble fraction of skin and liver (9, 10, 16). The first hypothesis is not consistent with the amount of cantharidin required to interfere with mitochondrial respiration *in vitro* (>4 mM) (11, 12) relative to its toxicity, and neither cantharidic acid nor endothal significantly alter mitochondrial $Mg^{2+}$-ATPase activity in mice either *in vivo* or *in vitro* (13). The second hypothesis is more consistent with the present study. The cytosolic fraction of skin pretreated with cantharidin induces acantholysis in untreated skin (16). The acantholytic factor appears to be a protein, based on dialysis and thermolability, and its activity is inhibited by sulfhydryl binding agents such as $HgCl_2$, $AgNO_3$, $As_2O_3$, and *N*-ethylmaleimide (9, 16). In contrast, *N*-ethylmaleimide does not interfere with [$^3$H]cantharidin binding. This difference can be rationalized on the basis that acantholytic activity results from a change in protein conformation on association of cantharidin with its binding site as suggested by its extremely slow rate of dissociation (17). Sulfhydryl binding agents may therefore act differently on the native protein and on that which has undergone a conformation change on combining with cantharidin. It is now important to isolate the cantharidin binding site and establish its relationship, if any, with the acantholytic factor and potential cytotoxic proteases (17).

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