hepatocyte co-cultures indicate that EE raised the amount of cytochrome P-450 and induced the formation of stable complexes to the same extent as *in vivo* after 3 days drug treatment of functional hepatocytes, without changes in its toxic effect. In the same conditions, EF did not induce cytochrome P-450 and did not give complexes. This agrees with preliminary *in vivo* findings. These data suggest that hepatotoxicity of erythromycin compounds is not due to prior oxidation of the macrolides.

Our observations are consistent with the statement of an intrinsic toxicity of erythromycin derivatives. However, the primary site of toxicity remains to be determined. This could be the plasma membrane since, in pure but not in mixed hepatocyte cultures, toxicity changed with culture time. Indeed, various alterations of the plasma membrane have been described not only during cell isolation but also in conventional culture [25]. In spite of evident similarities between *in vivo* and *in vitro* effects of erythromycin derivatives, further work is needed to ascertain that the same mechanism is involved in the two situations.

In conclusion, our observations with various liver cell cultures show that: (1) EF is less toxic than EB and EE and does not form stable and inactive complexes with cytochrome P-450; (2) EE is the most toxic erythromycin derivative and induces the formation of an inactive complex with cytochrome P-450. Its toxicity does not seem related to the cells' drug metabolizing capacity; (3) conventional and mainly mixed hepatocyte cultures are useful tools for pharmacotoxicological studies of erythromycin antibiotics and are to some extent predictive of the *in vivo* effects of these drugs.

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Thermodynamics of the interactions of tricyclic drugs with binding sites for [^{3}H]imipramine in mouse cerebral cortex

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The tricyclic antidepressant imipramine, an inhibitor of the neuronal uptake of serotonin, binds with high affinity to specific membrane-associated sites in the central nervous system [1, 2]. A large proportion of these binding sites are located on serotonergic nerve terminals [3, 4], and they recognize drugs with the same specificity as the transporter responsible for serotonin accumulation by nerve endings [5]. Most likely, imipramine and serotonin bind to the same molecular complex involved in serotonin uptake, but at distinct, allosterically interacting sites [6, 7]. Thus, tricyclic drugs would inhibit the uptake of serotonin by inducing conformational changes in the uptake complex [6]. In a

thermodynamic analysis, such conformational changes caused by binding may be shown to counteract the increase in entropy resulting from the displacement of structured water from hydrophobic groups on ligand and/or binding site [8–10]. Although there have been reports on effects of temperature on the binding of [^{3}H]imipramine to brain membranes [11, 12], a thermodynamic analysis has not been described yet. In the present study we have evaluated the thermodynamic parameters of the binding of [^{3}H]imipramine to membranes from mouse cerebral cortex between 3° and 25°.

Materials and methods

The following drugs were the donations of the companies indicated: amitriptyline, Merck Sharp & Dohme; chlorimipramine hydrochloride, Geigy Pharmaceuticals; cocaine hydrochloride, Mallinckrodt; desipramine hydrochloride, USV Laboratory; fluoxetine, Lilly Research Laboratories; imipramine hydrochloride, Geigy Pharmaceuticals; and norzimelidine, Astra. Serotonin hydrochloride was purchased from Sigma and *N*-[*methyl*-^{3}H]imipramine hydrochloride (75 Ci/mmole) from New England Nuclear.

Membranes from cerebral cortex of adult male BALB/cBy mice (20–25 g, Jackson Laboratories, Bar Harbor, ME) were prepared and assayed immediately for binding of [^{3}H]imipramine by the method of Langer *et al.* [5] as described previously [13]. The incubation mixtures contained 50 mM Tris–HCl, 100 mM NaCl, and 5 mM KCl, buffered at pH 7.4 at 25°. Binding assays were terminated by rapid filtration with a single-manifold Millipore filtration apparatus. Nonspecific binding of [^{3}H]imipramine (see below) contributed 20–32% of total binding. The IC_{50}* values of compounds in inhibiting the binding of [^{3}H]imipramine were calculated from log-probit analysis of inhibition obtained with four or five concentrations of the compound assayed in triplicate in three separate tissue preparations. K_I values were calculated from the IC_{50} values with the Cheng and Prusoff correction [14]: $K_I = IC_{50}/(1 + [L]/K_D)$. $[L]$, the concentration of free [^{3}H]imipramine, was 1.7 nM at 3°, 2.2 nM at 10° and 14°, and 3.0 nM at 17° and 25°. K_D values for [^{3}H]imipramine, determined by Scatchard analysis, were 21 nM at 3°, 41 nM at 10°, 91 nM at 17°, and 145 nM at 25°. K_D values for imipramine calculated from inhibition experiments (see Table 1) were similar. The standard Gibbs free energy change ($\Delta G°$) of binding was calculated for 25° from $\Delta G° = -RT\ln K_A$, in which R = gas constant (1.9872 cal/mole·deg), and $K_A = 1/K_I$. The standard change in enthalpy ($\Delta H°$) was obtained from the slope $-\Delta H°/R$ of the van't Hoff plot. The standard entropy change ($\Delta S°$) was calculated for 25° from $\Delta G° = \Delta H° - T\Delta S°$.

Results and discussion

To establish conditions that permit equilibration at the various temperatures, binding of [^{3}H]imipramine to membranes from cerebral cortex was examined as a function of time (Fig. 1). As the temperature was increased, the plateau of binding was reached more rapidly. In addition, with 4 nM [^{3}H]imipramine in the medium, the equilibrium binding decreased with higher temperatures, reflecting the decrease in affinity of the binding sites for imipramine (see also Fig. 2). For subsequent equilibrium binding measurements, the following incubation times were chosen: 60 min at 3°, 40 min at 10°, 32 min at 14°, and 25 min at 17° and 25°. The longer incubation time used at the lower temperatures did not by itself appear to change the properties of the binding sites, since the binding of [^{3}H]imipramine measured at 25° after preincubating the membranes for 1 hr at 3° was the same as the binding measured at 25° without the preincubation (data not shown). In addition, the binding measured at 3° after 25 min at 25° was the same as the binding at 3° to membranes not preincubated.

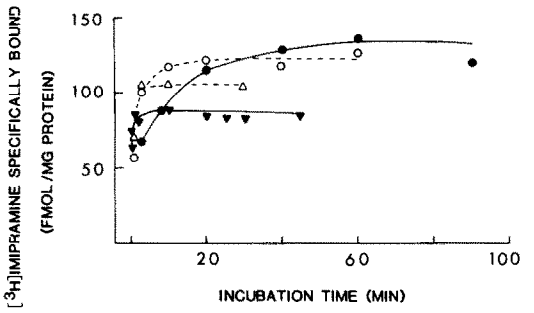

Fig. 1. Time course of binding of [^{3}H]imipramine in mouse cerebral cortex as a function of temperature. Washed membranes of cerebral cortex were incubated with 4 nM [^{3}H]imipramine for the times indicated. Each value shown is the mean of data obtained with three independent membrane preparations. Key: (●—●) 3°; (○—○) 10°; (△—△) 17°; and (▼—▼) 25°.

* Abbreviations: IC_{50}, concentration of a compound required to inhibit specific binding by 50%; K_A, equilibrium association constant; K_D, equilibrium dissociation constant; and K_I, equilibrium dissociation constant of inhibiting compound.

Table 1. Potencies of tricyclic and nontricyclic compounds in inhibiting [^{3}H]imipramine binding in mouse cerebral cortex*

	IC_{50}† (nM)				
	3°	10°	14°	17°	25°
Tricyclics					
Imipramine	26 ± 4	41 ± 9		77 ± 9	131 ± 24
Desipramine	220 ± 15	265 ± 35		400 ± 58	533 ± 120
Chlorimipramine	44 ± 7		138 ± 82		205 ± 40
Amitriptyline	75 ± 5		125 ± 15		300 ± 94
Nontricyclics					
Fluoxetine	102 ± 24	290 ± 20		750 ± 175	1,733 ± 809
Norzimelidine	131 ± 20	190 ± 20		1,275 ± 25	5,333 ± 952
Cocaine	2,532 ± 381		31,500 ± 18,500		713,333 ± 379,477
Serotonin	15,177 ± 4,206		90,000 ± 10,000		1,438,000 ± 84,716

* Results are mean values ± S.E.M. obtained from three independent membrane preparations (see text).
† Concentration of the compound that inhibits specific binding of [^{3}H]imipramine by 50%.

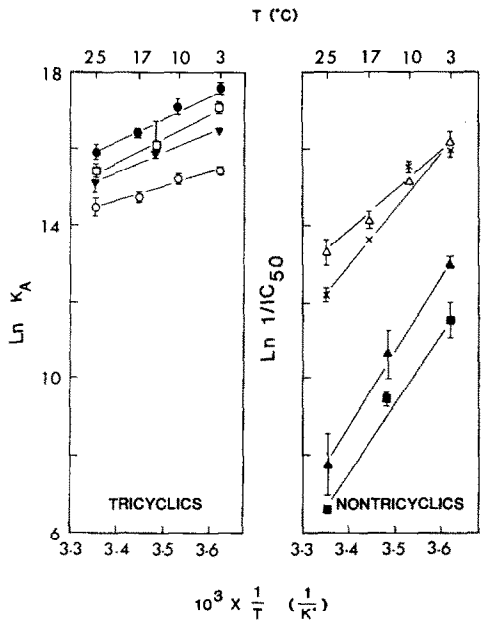

Fig. 2. Temperature dependence of the interactions of tricyclics and nontricyclics with the binding sites for [^{3}H]-imipramine in mouse cerebral cortex. The data for the tricyclic drugs (left panel) are shown as van't Hoff plots. For the nontricyclics (right panel), values of $1/IC_{50}$ are shown instead of values of K_A (see text). Each point is the average of data obtained with three independent membrane preparations, each assayed in triplicate. The S.E. (vertical bars) are indicated only if greater than the point itself. The straight line is the least square fit obtained from linear regression analysis. The correlation coefficients ranged from 0.81 to 0.97. Tricyclic drugs; (●) imipramine; (○) desipramine; (□) chlorimipramine, and (▼) amitriptyline. Nontricyclic compounds: (△) fluoxetine; (×) norzimeldine; (▲) cocaine; and (■) serotonin.

Since the affinity of the binding sites for tricyclics decreased with higher temperatures, we investigated whether these temperatures would require higher concentrations of desipramine for defining nonspecific binding. At all temperatures, the inhibition curve of [^{3}H]imipramine binding by desipramine showed a plateau around 4 and 10 μM. At 3° and 10° we used 4 μM desipramine for defining nonspecific binding; at higher temperatures we used 10 μM.

The effect of pH on specific binding of [^{3}H]imipramine was examined since the pH of the Tris buffer used showed some dependence on temperature: there was, at the most, a change of 0.2 pH units when the Tris, buffered at pH 7.4 at 25°, was cooled to 3°. However, both at 3° and at 25°, the specific binding of [^{3}H]imipramine showed a minimal pH dependence between pH 7.1 and 7.7 (data not shown). Similar findings were reported recently for imipramine binding at 4° to membranes of rat cerebral cortex [12].

In the following analysis we examined the equilibrium reaction of binding. It was not possible to evaluate the possible formation of an activated ligand–binding site complex based on the relation between temperature and the association and dissociation rate constants since, as shown in Fig. 1, the association rates were too high at temperatures above 10° to be measured accurately. The four tricyclic drugs imipramine, desipramine, chlorimipramine, and amitriptyline inhibited the binding of [^{3}H]imipramine to membranes from mouse cerebral cortex at all temperatures in a competitive manner with Hill coefficients close to unity (range 0.8 to 1.1). These interactions thus allow mass-action principles, allowing the use of the Cheng and Prusoff correction [14] for computing K_A values from IC_{50} values (Table 1), and allowing the calculation of thermodynamic parameters (Table 2) from the data presented in the van't Hoff plots (Fig. 2, left panel). The force of hydrophobic interactions originates in the removal of water from non-polar surface groups on ligand and/or receptor. Although the disruption of the "sweater" of water around the hydrophobic groups results in an increased entropy, other changes may occur that diminish or even reverse this increase, such as conformational changes or immobilization of receptor and ligand. This appears to be likely for the interactions of the tricyclics with the imipramine site in the present experiments, since the changes in entropy upon binding were small (desipramine) or even negative (imipramine, chlorimipramine, amitriptyline) as shown in Table 2. The observed entropy changes are consonant with the proposal [6] that the tricyclics induce conformational changes in the serotonin uptake complex. The changes in Gibbs free energy ($\Delta G°$ ranging from −8.6 to −9.4 kcal/mole) resulted almost entirely from the changes in enthalpy ($\Delta H°$ ranging from −7 to −12 kcal/mole) (Table 2), indicating an important enthalpic drive on the binding of tricyclic drugs to the [^{3}H]imipramine sites in the cerebral cortex. It should be kept in mind that the binding may be a multi-step process; the apparent K_A of binding may, in fact, not be a simple composite of the equilibrium constants of each individual step. However, in view of the linear van't Hoff plots, it seems reasonable to use the apparent K_A as an approximation of the true K_A [15]. In a different approach, we determined the hydrophobic sensitivity of the binding of [^{3}H]imipramine to a model lipid membrane, the liposome, and to brain membranes, by measuring the inhibition of binding by a series of drugs with different degrees of lipophilicity; in agreement with the present results it was concluded that hydrophobic forces do not drive the binding to brain membranes as they do in the case of the liposome [16].

Figure 2 (right panel) and Table 1 also show the temperature dependence of the inhibitory potencies of the nontricyclic compounds fluoxetine, norzimelidine, cocaine, and serotonin. It is obvious that the changes with tem-

Table 2. Thermodynamic parameters of binding of tricyclic drugs to imipramine recognition sites in mouse cerebral cortex*

	$\Delta G°$ (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$ (cal/mol·deg)
Imipramine	−9.4 ± 0.13	−11.5 ± 0.45	−6.8 ± 1.57
Desipramine	−8.6 ± 0.15	−7.0 ± 0.56	+5.5 ± 1.94
Chlorimipramine	−9.1 ± 0.11	−12.2 ± 1.24	−10.1 ± 4.18
Amitriptyline	−9.0 ± 0.19	−10.2 ± 0.94	−4.3 ± 3.23

* The thermodynamic parameters were calculated for 25° from the data shown in Fig. 2 as described in Materials and Methods. Values shown are the mean ± S.E.M. of three independent determinations. The S.E.M. values in $\Delta H°$ were obtained from linear regression analysis of the van't Hoff plot.

perature are far greater for these substances than for the tricyclics. Other evidence that the nontricyclics interact with the binding sites for [^{3}H]imipramine in a different manner comes from Hill analyses which indicate Hill coefficients between 0.50 and 0.58, appreciably lower than unity, for all temperatures studied between 3° and 25° (data not shown). Since the law of mass-action for the simple interaction of a homogeneous ligand with a single homogeneous site does not apply to the nontricyclic compounds, we did not attempt to calculate thermodynamic parameters for the nontricyclics. However, the intriguing possibility exists that the nontricyclics interact with the imipramine sites in a competitive manner, even though the Hill numbers are low, as is the case for inhibition of striatal [^{3}H]spiroperidol binding by dopamine [17]. Thus, the steeper plots for the nontricyclics (Fig. 2) could indicate that decreases in enthalpy are more important for the interactions of nontricyclic drugs than of tricyclic drugs; in addition, entropy changes could be more negative for the nontricyclics, possibly reflecting a more drastic conformational change induced by the nontricyclics than by the tricyclics. This could indicate a basic difference in the mechanism by which tricyclic and nontricyclic compounds affect the serotonin carrier complex and thereby regulate the uptake of serotonin into nerve endings. This interpretation is at variance with the idea of tricyclics and nontricyclics acting at distinct, allosterically interacting sites [6, 7], and it will require more experimentation to ensure that thermodynamic analysis can be applied to the data for the nontricyclics.

In conclusion, the present results indicate important differences between the interactions of tricyclic and nontricyclic compounds with high-affinity binding sites for [^{3}H]imipramine in the mouse cerebral cortex. The observed entropy and enthalpy changes are consonant with the proposal that tricyclics induce conformational changes in the serotonin uptake complex and that, finally, the enthalpy change is the force that drives the binding of tricyclic drugs to the [^{3}H]imipramine sites in the mouse cerebral cortex.

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