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Contribution of Aryl Groups to Toxic Interactions at Chemoreceptors: 3-Tropanol Phenylacetates and Their Hexahydro Derivatives as Toxic Agents[1, 2]

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Recent work with a series of monosubstituted compounds (Fig. 1) stemming from the phenylacetate esters I and I-ψ of the amino alcohols tropine (transoid) and ψ-tropine (cisoid) has shown that, in general, tropine aryl esters are more potent

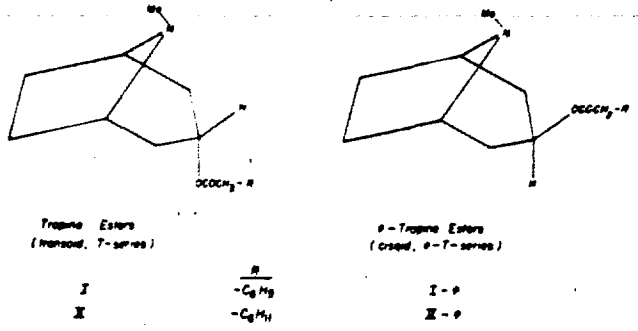

FIG. 1. Structures of 3-tropanol esters.

than their ψ-isomers in triggering a convulsion-paralysis-death syndrome in mice, and in evoking a concentration-dependent amplification of twitch response in the rat phrenic nerve-diaphragm preparation (Friess *et al.*, 1964a,b). These actions are stereospecific with respect to cisoid or transoid disposition of the —$OCOCH_2R$ residue on the tropane ring, and also very much dependent on the nature and position of single substituents (either electron-attracting or electron-donating) attached to the benzene ring of I or I-ψ. Such observations, together with the curious finding (Friess *et al.*, 1964b) that bulky ortho substitution in the aryl ring destroys the stereospecificity of the ester-receptor responses, are best accommodated by a model for receptor-ester binding which is multifunctional in character, with surface geometry of the receptor specifically tailored (or induced) to accommodate simultaneously the amine $>\overset{+}{N}HMe$, ester —OCO—, and aryl ring structures.

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However, at least one element of uncertainty in this model resides in the nature of the aryl group binding to the receptor surface, and particularly in the question of whether a charge-transfer process is invoked which imposes the *necessity* that the ring be aromatic. An answer to this question was sought directly by hydrogen saturation of the ring in I and I-ψ, yielding the cyclohexylacetate structures II and II-ψ with elimination of the conjugate unsaturation required for charge-transfer complex formation. The new saturated esters II and II-ψ have now been prepared and subjected to test on the same bioreceptor systems previously employed (intact animals, neuromuscular preparations, etc.) in observation of stereospecific effects produced by I and I-ψ, for pairwise comparisons of any differentiation in activity induced by saturation of the aryl ring. The somewhat surprising results of these comparisons form the basis of the present communication.

METHODS

Each of the new cyclohexylacetate esters II and II-ψ was synthesized by reflux of stoichiometric amounts of cyclohexylacetyl chloride and a given amino alcohol (tropine or ψ-tropine) in inert solvent, followed by isolation of the crude ester as its hydrochloride salt. The salts were repeatedly recrystallized to analytical purity in acetone–ether or chloroform–ether solvent mixtures, and submitted to elemental analyses, with the results summarized in Table 1.

TABLE 1
ELEMENTAL ANALYSES OF NEW ESTERS

Compound	Melting point (°C)	Analysis (%)		
		Element	Calculated	Found
II·HCl	225	C	63.66	64.19
		H	9.35	9.37
		N	4.64	4.38
II-ψ·HCl	201	C	63.66	63.59
		H	9.35	9.37
		N	4.64	4.56

Freshly prepared solutions of the cyclohexyl ester hydrochlorides in isotonic saline or in the appropriate buffer (Ringer) media were employed in toxicity studies with intact mice and cats, in work on twitch potentiation and twitch blockade with the excised phrenic nerve-diaphragm preparation (PN-D) from the rat, and in studies of *in vitro* inhibitory potencies with respect to the purified esterase system acetylcholinesterase–acetylcholine (AChE–ACh). The animals, techniques, and protocols employed were identical in strain and content with those described in previous work (Friess *et al.*, 1964a,b; Thron *et al.*, 1963), to ensure full comparability of results obtained with the presently studied cyclohexyl esters II and II-ψ, and the previous data on action spectra of the corresponding phenylacetates I, I-ψ. The AChE enzyme preparation used for the esterase inhibitory studies was a highly purified sample derived from electric eel tissue, its natural substrate acetylcholine ion was supplied to form steady-state reaction mixtures from freshly prepared solutions of doubly recrystallized acetylcholine chloride, and the enzyme kinetics of reactions involving

enzyme–substrate–inhibitor mixtures were followed by the pH-stat techniques previously described (Friess *et al.*, 1959).

RESULTS

Intravenous Toxicities in Mice and Cats

The new cyclohexylacetates II and II-ψ were tested as intravenously administered toxins in the NMRI strain of white mice (male), and in a mongrel population of well-nourished cats, with the results summarized in Table 2. These results are striking and in part quite unexpected, both from the standpoint of internal comparisons between the effects of II and II-ψ, and cross-comparisons with the corresponding aryl esters I and I-ψ (for which the mouse LD_{50} data are also recalled in the Table).

It is seen first from Table 2 that the esters II and II-ψ obey the same general toxicity pattern found previously for the arylacetic esters, with prominent features of transient paralysis, tonic and clonic convulsions, respiratory impairment, and other complex manifestations of neuromuscular involvement. At the quantitative level of comparison, in mice, the first unexpected finding emerges in the observation that stereospecificity in toxic response is lost in the cyclohexyl derivatives; II and II-ψ are equivalent in LD_{50} index of potency, within the spread of one standard deviation in the data. This finding is in strong contrast with all previous data from the aryl ester series (with the single exception of the class of bulky *o*-substituted aryl esters), in which the general rule has been a decided stereospecificity in toxic response with the invariant potency sequence: tropine ester > ψ-tropine ester (T > ψ-T). Further in the comparison of mouse toxicity data from Table 2, it is seen that hydrogenation of the phenyl ring in the potent T-series results in a very considerable drop in intrinsic toxicity (potency sequence I > II), whereas in the weaker ψ-T series saturation of the ring has only a marginal effect on potency (II-ψ ≧ I-ψ).

One finding in the cyclohexyl series which merits some emphasis, in contrast with the general aryl ester toxicity pattern, is the occurrence in the cat of a transient but marked impairment of visual function and some concomitant disorientation under the influence of the tropine ester II.

Effects on the Rat Phrenic Nerve-Diaphragm Preparation

The new cyclohexyl esters II and II-ψ afforded some surprises in their biphasic actions on the rat PN-D preparation, which turned out to be quite parallel to the unexpected facets of their toxic interactions in intact animals. The data are summarized in Table 3 in columns which deal, respectively, with the mode of electrical stimulation of the preparation (N = indirect stimulation via the phrenic nerve, M = directly via the muscle), the maximum degree of amplification produced by ester in isotonic N- and M-twitches at the 3-minute reference time, the concentration of agent yielding that maximum twitch potentiation, and the (higher) concentration of agent required to swamp out initial twitch amplification and produce 50% reduction from control twitch amplitude at the reference time post ester addition. Table 3 also reproduces reference data on twitch potentiation for the two phenyl esters I and I-ψ, for ease of comparison and contrast with results produced by the cyclohexyl esters II and II-ψ.

TABLE 2

ACUTE, INTRAVENOUS TOXICITIES OF TROPANOL ESTERS IN MICE AND CATS

Compound	Mice		Cats	
	LD_{50} (mg/kg)	Toxic signs	Dosage (mg/kg)	Toxic signs
II·HCl	36.0 ± 1.6	Immediate prostration, limbs extended, repetitive convulsive seizures, foreleg paralysis, loss of righting reflex. Later, broad-based stance, pawing at muzzle, hypersensitivity to auditory or tactile stimuli. Respiration impaired, frequent occurrence of tail curl, pronounced tendency to bite	30	Tonic and clonic convulsions, piloerection on back and tail, respiratory blockade with positive response to artificial respiration, gut contractures, mydriasis, powerful running motions during seizures. Partial crossing nictitating membranes. Recovery in minutes
			20–24	Broad-based sprawl, disturbance of muscular functions, whole-body tremors, mydriasis and temporary loss of vision, panting, tonic manifestations, disorientation. Recovers
II-ψ·HCl	34.0 ± 1.2	Signs as above. Additionally, respiration impaired by hiccoughs in large fraction of test animals, running and hopping, whole-body tremors, anoxia in later stages of repetitive convulsions	30	Rear limb paralysis, tonic and clonic convulsions, powerful arching of back, respiratory failure, death in 5 minutes
			20	Prostrate, limbs extended, gut contractures, piloerection, panting, hoarse cries, loss of foreleg control, tonic and clonic convulsions with back arched and running motions. Recovery over the course of an hour, with panting, yowls, excessive salivation, and periods of torpidity
I·HCl	23.0 ± 0.8[a]			
I·ψ·HCl	42.3 ± 1.6[a]			

[a] Friess *et al.* (1964a).

Referring to the twitch potentiation column of Table 3, and just to the peak percentage subcolumn for the clearest mode of comparison, it is seen that the cyclohexyl esters are once again distinguished by a failure to show stereospecificity in evoking their weak potentiating responses from the PN-D preparation, curarized or uncurarized. Esters II and II-ψ appear to be virtually equipotent in their feeble amplification of N- and M-twitch responses, just as they are equipotent with respect to lethal strength in the intact mouse. Further, and in exact continuation of the analogy with regard to LD_{50} levels in the mouse, the comparison of phenyl *vs.* cyclohexyl ester potentiating strength shows the stereospecific power sequence I > II in the stronger T-series, whereas in the weaker ψ-T series this stereospecificity vanishes

TABLE 3
EFFECTS OF TROPANOL ESTERS ON RAT PHRENIC NERVE-DIAPHRAGM PREPARATION

Compound	Stimulation pathway	Potentiation[a] Control at peak (%)	Potentiation[a] Concentration at peak (*M*)	BC_{50}[d] (*M*)
II	N	122 ± 3	$3.0 \pm 0.2 \times 10^{-4}$	$9.2 \pm 0.3 \times 10^{-4}$
	M	113 ± 5	$3.2 \pm 0.2 \times 10^{-4}$	$1.8 \pm 0.1 \times 10^{-3}$
II + *d*-tubo[b]	M	130 ± 4	$5.2 \pm 0.2 \times 10^{-4}$	$2.2 \pm 0.1 \times 10^{-3}$
II-ψ	N	124 ± 5	$2.2 \pm 0.2 \times 10^{-4}$	$6.3 \pm 0.2 \times 10^{-4}$
	M	114 ± 4	$2.0 \pm 0.2 \times 10^{-4}$	$1.2 \pm 0.1 \times 10^{-3}$
II-ψ + *d*-tubo[b]	M	130 ± 6	$3.3 \pm 0.3 \times 10^{-4}$	$8.8 \pm 0.3 \times 10^{-4}$
I[c]	N	142	5.4×10^{-4}	
	M	142	5.8×10^{-4}	
I-ψ[c]	N	120	5.6×10^{-4}	
	M	125	5.5×10^{-4}	

[a] All readings of twitch height relative to control levels were made at 3.0 minutes after drug addition.

[b] Preparation curarized at the 1.0×10^{-5} *M* level of *d*-tubocurarine chloride.

[c] Data from the paper by Friess *et al.* (1964a).

[d] Concentration resulting in 50% reduction of twitch amplitude (or twitch tension) at the 3.0-minute time after ester addition.

into a rough power equivalence I-ψ ≈ II-ψ for the two weak potentiating agents. In general with this excitable preparation the effect of hydrogenating the phenyl group in I and I-ψ seems to be an abrupt diminution in the power of the T-isomer down to the potency level of the weaker ψ-T ester, with a simultaneous loss of the stereospecificity (T *vs.* ψ-T) inherent in the aryl esters.

Effects on the Acetylcholinesterase–Acetylcholine System

In view of the curious finding in the mouse toxicity and PN-D potentiation studies that hydrogenation of the phenyl group abruptly wipes out stereospecificity in biological response and reduces the absolute level of the response to that of the ψ-series, it became a matter of immediate interest to assay the new cyclohexyl esters for their inhibitory potencies *vs.* the ***in vitro*** AChE–ACh system operating at steady state. This esterase system has consistently shown a ψ-T > T ester stereospecificity in

inhibitory strength for every pair of aryl ester isomers tested to date. The results of kinetic tests of the cyclohexylacetate esters II and II-ψ as reversible inhibitors of the esterase are briefly summarized in Table 4. The data in Table 4 are presented in the form of enzyme-inhibitor complex dissociation constants K_I, calculated on both the competitive and noncompetitive bases by the equations of Wilson (1949). Constancy of a given inhibitory mechanism within a limited substrate concentration range, as indicated by braces around the data groupings of Table 4, is inferred from the constancy of calculated K_I values as the initial substrate concentration is varied.

TABLE 4
ACTIVITIES OF TROPANOL ESTERS AS ACHE-ACH INHIBITORS

Compound	Initial ACh concentration (mM)	Inhibition[a] K_I (comp)	Inhibition[a] K_I (non-comp)
II	3.33	$3.55 \pm 0.10 \times 10^{-5}$	$5.73 \pm 0.15 \times 10^{-4}$
	2.50	$3.49 \pm 0.08 \times 10^{-5}$	$4.31 \pm 0.10 \times 10^{-4}$
	1.67	$4.07 \pm 0.14 \times 10^{-5}$	$3.49 \pm 0.12 \times 10^{-4}$
II-ψ	3.33	$8.38 \pm 0.16 \times 10^{-6}$	$1.35 \pm 0.03 \times 10^{-4}$
	1.67		$1.22 \pm 0.04 \times 10^{-4}$
I[b]	3.33	$1.46 \pm 0.06 \times 10^{-4}$	$2.36 \pm 0.10 \times 10^{-3}$
	1.65		$2.31 \pm 0.10 \times 10^{-3}$
I-ψ[b]	3.33	$7.20 \pm 0.13 \times 10^{-5}$	$1.16 \pm 0.02 \times 10^{-3}$
	1.67	$7.29 \pm 0.22 \times 10^{-5}$	

[a] At pH 7.40 and 25.21 ± 0.04° C.
[b] Data from paper by Friess *et al.* (1964a).

With decreasing K_I values indicative of increasing inhibitory potency, and using the K_I (comp) values at the 3.33 mM level of ACh as the reference activity index, it is seen from Table 4 that this enzyme shows a sharply stereospecific preference for II-ψ over II as an inhibitor (by a strength factor of 4). This structural preference is in the normal steric sense for this enzyme (ψ-T > T). Further, the effect of hydrogenation of the phenyl ring is directly opposed to that seen in mice and with the PN-D preparation; saturation of the ring in each series leads to an *increase* in AChE-inhibitory potency (strength sequences: II > I by a factor of 4; II-ψ > I-ψ by a factor of 8.6).

A further item of interest from Table 4 stems from the similarity in the pairs II, II-ψ and I, I-ψ with respect to failure to adhere to a single inhibitory mechanism. It is seen that both the cyclohexylacetates and the phenylacetates undergo a change in inhibitory mechanism on shift from one stereochemical series (T) to the other (ψ-T).

DISCUSSION

The present results offer an exceptional demonstration of the role played by an aryl group in adding to effective interactions at biological chemoreceptors in tissues. The essential elements of interacting structure in the triggering molecules I, I-ψ, II and II-ψ group into the categories (a), (b) and (c), in which (a) and (b) are frozen geometrically (Fig. 2) into either T or ψ-T configurational relationship, and

in which T-geometry is favored by tissue receptors responsive to aryl esters. Now, the extent to which aromaticity and/or planarity in structural element (c) aids the total interaction process in tissue leading to receptor-controlled responses seems to depend intimately on the nature of the receptor involved. With receptors involved in the paralysis-convulsion toxicity syndrome in mice, and with those controlling twitch potentiation in the rat PN-D preparation, saturation in the phenyl ring of T-series esters results in an abrupt *decrease* in trigger potency (I *vs.* II), and therefore presumably a corresponding decrease in the contribution made by structure element (c) to receptor interaction processes. This decrease in potency is only observed when structural elements (a) and (b) are in the favored transoid (T-series) relationship with respect to one another, and the decrease at most is only down to the strength level characteristic of the aryl ester with the unfavored cisoid relationship between (a) and (b).

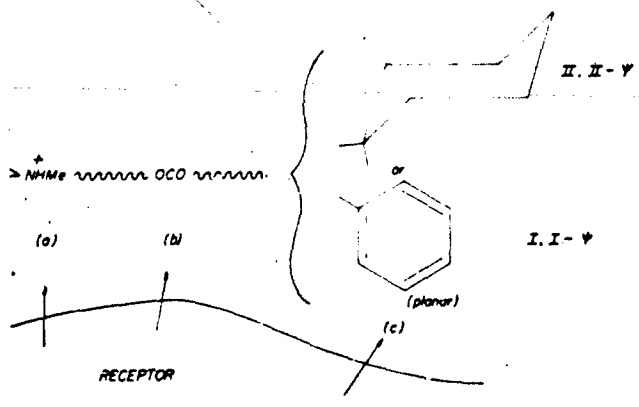

FIG. 2. Elements of structure affecting ester interaction with receptor sites.

Therefore, it would appear that the planar aryl group at (c) does indeed contribute to the total interaction potency of the T-series phenyl ester I at these particular tissue receptors, and that the structural transformation phenyl → cyclohexyl wipes out this increment of potency. Further, the fact that this increment occurs only in the stereochemically favored T-series and not with ψ-T isomers implies that the drug receptors triggered by these esters show a geometric specificity that is at least threefold. Only when (a) and (b) are bound in transoid fashion can the aryl group be brought into proper juxtaposition with the receptor surface for the additional interaction (c) as the third mode of effective contact. The net demonstration of selectivity on the part of (central) receptors in the mouse and peripheral synaptic receptors of the rat, then, is a show of enhanced susceptibility to the action of I [modes (a), (b) and (c) all operative], and a lesser (and constant) level of response to esters I-ψ, II and II-ψ in which only modes (a) and (b) contribute to interaction with receptor surfaces.

There is also a reciprocal implication in the potency equivalence II ≡ II-ψ with these two classes of receptors. Unless an aryl group is present per se to add its increment of interaction at the receptor surface, the receptor loses its stereoselectivity with respect to discrimination between cisoid and transoid ester configurations at

(a)-(b), and lumps all bipolar modes of attachment into one activity level. But when mode (c) is brought to bear, selectivity in terms of favoring the transoid mode at (a)-(b) is apparent. From this point of view, stereospecificity in these tissue responses is only unlocked by the necessary condition that a phenyl (or substituted phenyl) group add increment (c) to the interaction picture.

On shifting to the class of "receptors" exemplified by the active site region of the enzyme AChE, however, a radically different ester interaction picture from that drawn above emerges. The inhibitory strength sequences II-ψ > II and I-ψ > I (Table 4) clearly show the superiority of cisoid structure over transoid configuration in AChE inhibition, independent of any requirement for the presence of a phenyl group in the inhibitor. Indeed, in the full sequence of four esters in Table 4, the intrinsic inhibitory potency runs in the series: II-ψ > II > I-ψ > I. Therefore the cyclohexyl derivatives are both more potent than their aromatic phenyl congeners, and no phenyl group is required as a necessary condition for evocation of T *vs.* ψ-T stereospecificity. Further, the comparative findings that (1) the specificity pattern for AChE inhibition is exactly inverted from that shown by the mouse and rat chemoreceptors, and (2) the esterase shows no requirement for phenyl group substitu-

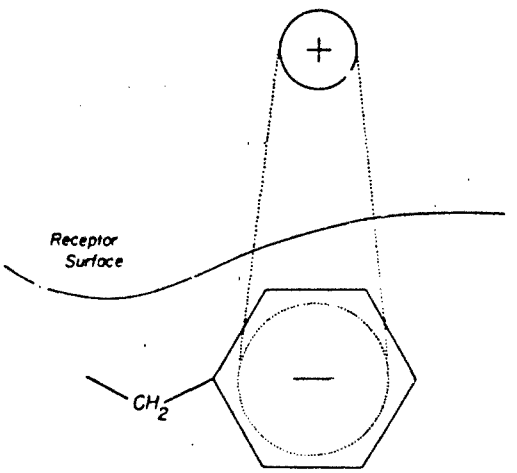

FIG. 3. Potential charge-transfer complex formation during ester-receptor interaction.

tion as a prerequisite to stereospecificity whereas the animal tissue receptors do, both militate against the possibility that this esterase moiety is an integral part of the animal chemoreceptor systems studied.

The observation that the cyclohexyl esters are respectively stronger than their phenyl analogs in inhibition of the AChE-ACh system *in vitro* is also subject to one further line of interpretation. If the primary enzyme-inhibitor binding is of the binary type involving simultaneous interactions at anionic and esteratic sites of the catalytic locus (Nachmansohn and Wilson, 1951), then any further diversionary modes of ester attachment to the catalytic surface could act to the detriment of the primary binding modes. In particular, if phenyl groups are bound at a third point on or near the catalytic locus and cyclohexyl groups are not, this extra binding could

well disrupt the precise positioning and interaction at the anionic-esteratic region (via $>\overset{+}{N}HMe$ and —OCO— functions in the ester) and produce the drop to weaker inhibition characteristic of the phenyl esters I and I-ψ.

One final point of interest that attaches to the finding of a phenyl group requirement [mode (c)] for enhanced toxicities in the mouse and potentiating activity at the rat PN-D synapse centers about possible mechanisms for modes of interaction in the tissue milieu. Since the net steric bulk of a phenyl residue is not too different from that of its hexahydro derivative, it would seem that planarity and conjugate unsaturation in the phenyl ring hold the key to a feasible mechanism underlying the mode (c) contribution to ester-receptor interaction. These two features of aryl group structure, of course, are precisely suited to the general phenomenon of complex formation via a charge transfer process, in which the phenyl group acts as an electron sink. Hence, a charge-transfer complex of the type shown in Fig. 3 might well account for the added increment of interaction effectiveness afforded by aromaticity in tropine esters based on structure I.

SUMMARY

The new cyclohexylacetate esters of tropine and ψ-tropine have been studied as toxins by the intravenous route in intact mice and cats, as twitch-potentiating agents on the rat phrenic nerve-diaphragm preparation, and as inhibitors of the acetylcholinesterase-acetylcholine system. In sharp contrast with their phenyl ester analogs, the cyclohexyl esters show an abrupt loss of capability to evoke *stereospecific* responses from chemoreceptor systems in the rat and mouse tissues, and yet display absolute levels of activity equivalent to those of the phenyl ester in the weaker ψ-tropine series. With the acetylcholinesterase system studied as a key nerve enzyme, the cyclohexyl esters show exactly inverted behavior in the sense that they are highly stereospecific with respect to their inhibitory potencies (ψ-tropine > tropine ester), and each cyclohexyl ester is significantly stronger as a reversible inhibitor than its phenyl analog.

These results are interpreted in terms of possible modes of functional interaction between these esters and the chemoreceptors controlling the responses of the tissue and enzyme systems studied.

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