Mode of Action of Nicotine in the Housefly

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The binding of H^3-nicotine to extracts of housefly heads has been examined and compared with the binding of H^3-muscarone. Both bind reversibly to the same protein(s) which appears to be the acetylcholine receptor(s). There is a good correlation between the toxicity of nicotine and five analogs to houseflies, and their ability to bind with the receptor(s). It is therefore suggested that combination with the receptor is the cause of the toxic action of nicotine and related compounds.

In 1857 Bernard first showed that in the vertebrate, nicotine acted on the autonomic nervous system. In 1906 Langley demonstrated that it also acted upon the skeletal muscle of vertebrates. He postulated that in both cases nicotine acted upon a "receptive substance," now called "receptor"; it is now recognized, on physiological evidence, that the acetylcholine receptor is the site for nicotine action in vertebrates (Trendelenberg, 1965; Volle and Koelle, 1969).

As for invertebrates, the earliest study was that of Greenwood (1890), who carried out a comparative study on the action of nicotine on several invertebrate groups and concluded that "the toxic effect of nicotine on any organism is determined mainly by the degree of development of the nervous system." Several workers (Hockenyos and Lilly, 1932; McIndoo, 1937; Yeager and Munson, 1942) found that the paralytic action of injected nicotine in insects was faster and toxicity was higher when the point of injection approached the central nervous system, thus reinforcing the view that the target lies within the central nervous system. Electrophysiological studies of the effect of nicotine on the ganglia of the cockroach, *Periplaneta*, and the locust *Locusta migratoria*, showed that it had a similar effect as it did on vertebrates: at low dosages it was stimulatory and at higher ones it was inhibitory (Harlow, 1958; Roeder and Roeder, 1939). Interestingly, transmission block was recently found in *Periplaneta* to be reversible in the presence of nicotine (Flattum and Sternburg, 1970a).

By analogy with its mode of action in vertebrates, and because of its ineffectiveness on insect cholinesterases (Richards and Cutkomp, 1945), it has been suggested that nicotine acts on the acetylcholine receptor. Yamamoto *et al.* (1962) studied the toxicity-structure relationship of 26 synthetic nicotine analogs and found that those bearing resemblance in configuration and charge distribution to acetylcholine were toxic to several insect species; he concluded that nicotine reacted with the acetylcholine receptor.

All the investigations conducted on the mode of action of nicotine have made use of toxicological or electrophysiological approaches, utilizing either whole animals or organs. Recently, however, a new direct method for such studies was reported, utilizing the *in vitro* binding of drugs (O'Brien and Gilmour, 1969; O'Brien *et al.*, 1970). Eldefrawi and O'Brien (1970) showed that the supernatant of 100,000 × g of the aqueous brain extract of the housefly, *Musca domestica* L., contained macromolecules that bound H^3-muscarone [an acetylcholine agonist (Waser, 1960)] with a high affinity, and were blocked by cholinergic drugs; these appeared to be the acetylcholine receptors. Such information was utilized in the present investigation to study the interference with H^3-muscarone and also H^3-nicotine binding by noncholinergic and cholinergic drugs, including nicotine and its toxic and nontoxic analogs. This represents a direct test of the mode of action of nicotine in the housefly. In addition, by comparing such results with those found for muscarone, the identity of the acetylcholine receptor molecules in the preparation may be better understood.

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MATERIALS AND METHODS

The extraction of the brain of the housefly (Wilson strain), H^3-muscarone, and equilibrium dialysis (the method used for studying binding), as well as the source and treatment with enzymes have previously been described (Eldefrawi and O'Brien, 1970). Two independent experiments, five replicates each, were conducted for each point, and the binding study was done exclusively on the supernatant of 100,000 × g of the housefly brain, at the concentration of 200 mg of heads per ml.

H^3-Nicotine (specific activity 300 mC/mM) was purchased from Amersham-Searle (Arlington, Ill.). Chromatography of the radiolabeled nicotine on I.T.L.C. media (Gelman Instrument Co., Ann Arbor, Mich.) revealed the presence of a slower moving component, which represented 15% of the total radioactivity. No identification attempts were made on this impurity. Comparisons were made of isolated impurity, pure nicotine, and the impure material with respect to the amount of binding at 10^{-6} *M*. They were not distinguishable. 3-Pyridylmethyl-*N*-dimethylamine, 3-pyridylmethyl-*N*-diethylamine, and *N*-(3-pyridylmethyl) morpholine were kindly donated by Izuru Yamamoto, and were stored at 2° C.

The toxicity of nicotine and its analogs was determined by topical application of 2λ of an ethanolic solution on the abdomen of 4-day-old unsexed, CO_2 anesthetized houseflies of the susceptible Wilson strain. Four replicates of 25 houseflies were made for each concentration, and mortalities were recorded 24 hr later.

The anticholinesterase activity of nicotine and its analogs on acetylcholinesterase of the housefly brain was determined by the titrimetric method using a Radiometer pH-stat, 6.25 mM NaOH as a titrant, and acetylcholine (3 mM) as a substrate, at pH 7 and 25° C.

RESULTS

In a previous study our data suggested that H^3-muscarone bound to acetylcholine receptor molecules present in the supernatant of 100,000 × g, 1 hr of the housefly head (Eldefrawi and O'Brien, 1970). Acetylcholine agonists and antagonists must bind to the acetylcholine receptor, and there-

Table I. Blockade of H^3-Muscarone Binding (at $10^{-6}M$) to Macromolecules in the Supernatant (100,000 × g, 1 hr) of Housefly Brains by Nicotine Analogs, and Their Toxicity to Houseflies

Nicotinoid	% Blockade $10^{-5}M$	% Blockade $10^{-4}M$	LD_{50} mg/kg
Nicotine	50	100	250[b]
Anabasine	25	98	200
3-Pyridylmethyl-dimethylamine	45	92	800
3-Pyridylmethyl diethylamine	61	97	550
N,N-Diethylnicotinamide	...	0	5000
N-(3-Pyridylmethyl)-morpholine	...	(8)[a]	5000

[a] Values in parentheses were less than two standard deviations different from controls and were considered not significant. [b] The LD_{50} dose for nicotine, if all penetrated and none were lost, would be $1.6 \times 10^{-3}M$ if uniformly distributed in a 20 μl fly.

Table II. Parameters Computed from the Lineweaver-Burk Plots for the Binding of Muscarone and Nicotine to the Supernatant (100,000 × g, 1 hr) of Housefly Brain

Ligand	Binding Constant $K(10^{-6}M)$	Maximum Binding R(nMoles/g heads)
Muscarone	2.4 ± 0.37	3.2 ± 0.27
Nicotine	3.22 ± 1.7	2.25 ± 1.05

fore should block muscarone binding. Nicotine, anabasine, and two toxic and two nontoxic synthetic analogs were selected to study the relation between their toxicity and their binding to the acetylcholine receptor. Only the toxic compounds interfered and blocked muscarone binding (Table I); and as expected, the higher concentrations ($10^{-4}M$) gave higher effects.

The binding of H^3-nicotine to macromolecules in the supernatant (100,000 × g, 1 hr) was also studied. The amount bound at $10^{-6}M$ was found to be a linear function of the amount of tissue extracted in the supernatant (Figure 1). The binding affinity for nicotine was determined by studying the extent of binding as a function of concentration. From the Lineweaver-Burk plot (Figure 2), the binding constant K and maximal binding R were computed by the weighted regression method of Wilkinson (1961). Nicotine and muscarone showed remarkably similar affinities and maximal binding (Table II).

The reversibility of nicotine binding was studied by placing four dialysis bags, each containing 1 ml of the supernatant fluid fraction, in 100 volumes of $10^{-6}M$ nicotine Ringer solution. Nicotine binding was then determined in five replicates from each of two bags, while the other two bags were redialyzed in 100 volumes of Ringer free of nicotine, and the extent of nicotine binding was determined the following day. Of the 0.64 nmoles of nicotine per g heads bound after the initial dialysis, 84% was removed by subsequent dialysis against 100 volumes of nicotine-free Ringer. Expected removal for total reversibility is 98.5%, as computed from an extrapolation of the data of Figure 2.

Blockade of nicotine binding by two noncholinergic and six cholinergic drugs, including nicotine analogs, was studied (Table III). Except for the nontoxic nicotine analog, *N*-(3-pyridylmethyl) morpholine, only the cholinergic drugs blocked nicotine and muscarone binding, while the noncholinergic serotonin and γ-aminobutyrate had no effect. Their blocking effects were similar on both ligands, except for 3-pyridylmethyl dimethylamine, which blocked muscarone binding rather more than nicotine; but the effect was of only modest significance ($P < .05$, $> .01$).

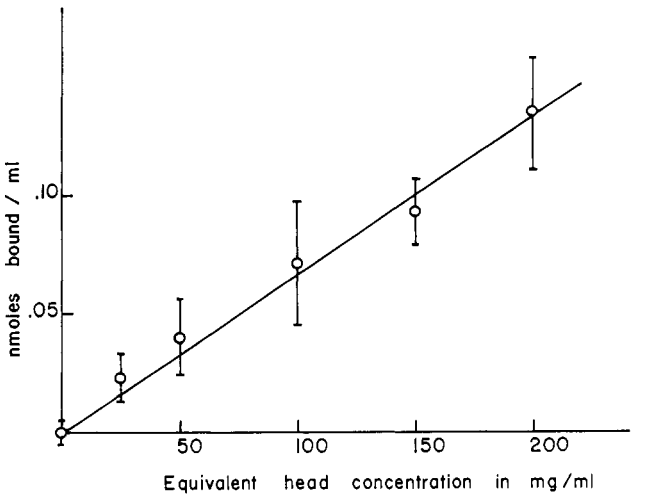

Figure 1. Binding of nicotine to the supernatant fluid fraction (isolated from heads of houseflies at 100,000 x g for 60 min) as a function of the weight of housefly heads used as a source for the isolation. Vertical lines represent the standard deviation for the 10 replicate experiments

The effect of preincubation (1 hr) of the supernatant with various enzymes (1 mg per ml of supernatant) was studied. Lipase (E.C. 3.1.1.3), *Crotalus adamanteus* venom (source of phospholipase A, E.C. 3.1.1.4), and phospholipase C (E.C. 3.1.4.3, from *Clostridium perfringens*), had no effect; but trypsin (E.C. 3.4.4.4) and chymotrypsin (E.C. 3.4.4.5) reduced binding of nicotine significantly (Table IV). This suggested that the binding macromolecules were proteins. However, trypsin and chymotrypsin reduced muscarone binding significantly ($P < .01$) more than nicotine. Probably enzymatic degradation produces materials which bind differentially to muscarone and nicotine.

Preincubation (10 min) of the housefly head supernatant with $10^{-4}M$ nicotine or any of its toxic or nontoxic analogs

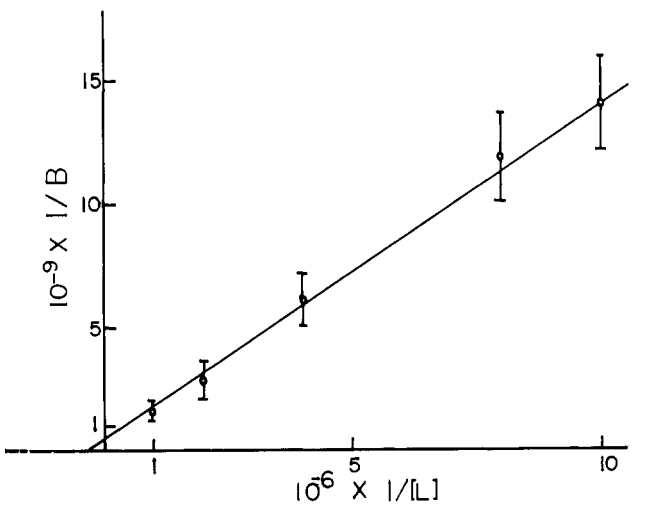

Figure 2. Lineweaver-Burk plot of the binding of nicotine (B; in moles/g of heads) to the supernatant fluid fraction, as a function of the concentration of nicotine (L; molar conc.). The weighting of the points varies because of the double-reciprocal nature of the plot; consequently visual fitting is inappropriate. The plotted slope was computed from all points by the weighted regression method of Wilkinson (1961). Vertical lines represent the standard deviation for 10 replicates at each concentration

Table III. Blockade of H^3-Nicotine and H^3-Muscarone Binding (at $10^{-6}M$) by Drugs (at $10^{-4}M$)

Drug	% Blockade	
	Nicotine	Muscarone
Cholinergic		
Decamethonium	75	76
d-Tubocurarine	55	54[a]
3-Pyridylmethyl-dimethylamine	66	92
N-(3-Pyridylmethyl)-morpholine	23	(8)[b]
Atropine	80	72[a]
Pilocarpine	84	84[a]
Noncholinergic		
Serotonin	(15)[b]	(13)[a,b]
γ-Aminobutyrate	(5)[b]	(−7)[a,b]

[a] From Eldefrawi and O'Brien (1970). [b] Negative values indicate greater binding in the presence of the drug. Values in parentheses are less than two standard deviations different from controls and were considered not significant.

Table IV. Effect of Enzyme Treatment on Nicotine Binding by the Supernatant (100,000 × g, 1 hr) of Housefly Brain

Enzyme	% Reduction of Binding	
	Nicotine	Muscarone
Trypsin	56	85
Chymotrypsin	55	79
Phospholipase A	(11)[a]	(12)
Phospholipase C	(−4)	(5)
Lipase	(16)	(3)

[a] Values in parentheses were less than two standard deviations different from controls and were considered not significant.

resulted in no reduction of acetylcholinesterase activity, as measured by the pH-stat.

DISCUSSION

The first part of this discussion is designed to show that nicotine binds to the acetylcholine receptor in the housefly, and that this binding is the cause of, or is directly related to, its insecticidal action.

Evidence has been provided that the binding of muscarone in the $10^{-6}M$ range to supernatants of housefly head preparations is to acetylcholine receptor (Eldefrawi and O'Brien, 1970). The present paper suggests that nicotine and muscarone bind to the same macromolecules: the number of binding sites for both is not significantly different (Table II); the response of both to eight blocking agents is similar (Table III); the binding molecules for both are sensitive to trypsin and chymotrypsin, but not phospholipase C (Table IV); and the binding of both is reversible. It seems probable, therefore, that nicotine (like muscarone) binds to the acetylcholine receptor.

Confirmatory evidence is the parallelism between the *in vitro* data provided above and established physiological facts. Thus drugs which have potent action physiologically on cholinergic systems also block muscarone binding *in vitro* (Table III), whereas noncholinergic agents such as serotonin and γ-aminobutyrate have no blocking action. In addition, nicotine's physiological effects (Harlow, 1958; Roeder and Roeder, 1939) and *in vitro* binding are both reversible.

Finally let us consider the relation between binding and toxicity. Table I shows parallelism between the binding of six nicotine analogs to housefly head supernatants (as judged by their relative potencies in displacing H^3-muscarone) and their toxicities to houseflies: toxic compounds bind and nontoxic ones do not. But within the toxic group, the correlation of binding potency to toxicity is not very good. However, toxicity is the final outcome of several factors acting together (rates of penetration through cuticle and to target, metabolism, excretion, storage, and action on target); and binding to the acetylcholine receptor is only one.

Recently, from electrophysiological data on transmission in the sixth abdominal ganglion of *Periplaneta*, it was suggested that different types of synaptic receptors were present, and that in addition a part of the total effect of nicotine might be mediated through the release of synaptically active material (Flattum and Sternburg, 1970a,b). Our findings do not exclude these possibilities. There could be more than one receptor, which might not be resolved under our conditions; or additional receptors may exist, not detected under our conditions.

Relation to Acetylcholinesterase. Two quite different steps are involved in the action of acetylcholine as a transmitter in the nervous system: the triggering action of acetylcholine on the receptor, and the subsequent destruction of acetylcholine by the enzyme acetylcholinesterase.

Though the latter has been purified and studied extensively, the receptor has not yet been isolated in pure form. It has been suggested that the receptor is nothing but the acetylcholinesterase macromolecule. It has also been suggested that its physiological function is due to the catalytic center of the enzyme (Ehrenpreis, 1967; Wurzel, 1967), only the anionic site of the catalytic center (Zupancic, 1967), both this anionic site plus an additional peripheral one (Changeux, 1966), or maybe only the latter (Podleski, 1967; Changeux *et al.*, 1969). Yet much evidence is accumulating to suggest that the receptor and the enzyme are separate molecules (Bartels and Nachmansohn, 1969; Karlin, 1967; Karlin and Bartels, 1966; O'Brien and Gilmour, 1969; O'Brien *et al.*, 1970). The present findings that nicotine or its analogs at $10^{-4}M$ had no inhibitory effect on acetylcholinesterase activity, but bound to and blocked the binding of muscarone to the receptor (Tables I, III), prove that they are not interacting with the catalytic site of acetylcholinesterase at this or lower concentrations. Fujita *et al.* (1970) noted that acetylcholinesterase of housefly head could be inhibited only by extremely high concentrations of nicotine and its analogs in the range of 1–1000 mM.

Practical Significance. Currently, nicotine and its analogs and Cartap (Sakai, 1970) are the only insecticides which act upon the acetylcholine receptor. A better knowledge of this action may lead to new kinds of insecticidal receptor agents. It is especially noteworthy that the housefly acetylcholine receptor may have unusual properties. Based upon the physiological action of drugs in vertebrates, cholinergic receptors are classified into muscarinic and nicotinic types. Nicotine is strictly nicotinic, but muscarone (unlike muscarine) acts on both types of receptors. According to the effect of drugs on the binding of muscarone (Eldefrawi and O'Brien, 1970) (Table I) or nicotine (Table III), the housefly brain exhibited both nicotinic and muscarinic character. Because of the similarities in the binding characteristics of nicotine and muscarone, the equal number of receptors involved (Table II), and blockade of binding by both nicotinic (*d*-tubocurarine and decamethonium) as well as muscarinic (atropine and pilocarpine) drugs, it is suggested that the acetylcholine receptor molecules of the housefly brain are of one type, having both muscarinic and nicotinic characters, rather than being of two separate types—either muscarinic or nicotinic. In this manner, they would resemble acetyl-

choline receptors of some species of crayfish (McLennan and York, 1966). It is therefore possible that one might design toxic agents which could discriminate between insect and vertebrate receptors.

Apart from the reception and hydrolysis of acetylcholine, other macromolecules are involved with the synthesis, storage, and release of acetylcholine. These present vulnerable targets which should be explored.

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