(1970).
(14) P. Portoghese, *Annu. Rev. Pharmacol.*, **10**, 51 (1970).
(15) A. R. Main and F. Iverson, *Biochem. J.*, **100**, 525 (1965).
(16) H. J. Schaeffer, M. A. Schwartz, and E. Odin, *J. Med. Chem.*, **10**, 686 (1967).
(17) D. J. Triggle, *Advan. Drug Res.*, **2**, 171 (1965).
(18) M. May, J. F. Moran, H. Kimelberg and D. J. Triggle, *Mol. Pharmacol.*, **3**, 28 (1967).

Axonal Cholinergic Binding Macromolecule. Response to Neuroactive Drugs†

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The interactions between the axonal cholinergic binding macromolecule, obtained from the walking leg nerves of the lobster, *Homarus americanus*, and several pharmacological agents which block the conduction of an axonal action potential have been studied. Those compounds which were competitive inhibitors of [^{3}H]nicotine binding included tetraethylammonium bromide ($K_i = 1.6 \pm 0.1 \times 10^{-5} M$) which blocks the increase in K^+ conductance only when applied inside of the lobster axon membrane and procaine ($K_i = 2.9 \pm 0.2 \times 10^{-6} M$) and hemicholinium 3 ($K_i = 2.8 \pm 0.2 \times 10^{-5} M$) which have local anesthetic effects and also act on the inner surface of the membrane. Ouabain was a noncompetitive inhibitor of [^{3}H]nicotine binding with a $K_i = 7.0 \pm 0.6 \times 10^{-5} M$. However, kinetic studies failed to indicate any interaction between nicotine and the Na^+K^+-ATPase. All the results still support the hypothesis that the axonal cholinergic binding macromolecule is on the internal surface of the axon plasma membrane and may be a component of both the Na^+ and K^+ gates.

Several pharmacological agents are now known which block the conduction of an axonal action potential by mechanisms which have been determined by physiological experiments.[1] It should be feasible to study the binding of such compounds to axon membranes in an attempt to identify and characterize the macromolecular components essential for axonal conduction. Such an approach is analagous to that being used to characterize postsynaptic cholinergic receptors.[2] Alternatively, it is also possible to study the interactions of these drugs and toxins with enzymes and macromolecules known to be present in the axon membrane in an attempt to assign a role to them in the conduction of an action potential. This has recently been done by Matsumura and Narahashi[3] who investigated the function of the ATPases in axonal conduction in lobster nerves.

Recently, we described a macromolecule present in an axon plasma membrane preparation from lobster walking legs which binds cholinergic ligands and local anesthetics.[4a] We shall call this the axonal cholinergic binding macromolecule (ACBM). A cholinergic receptor in the axon has long been predicted by Nachmansohn[5] who hypothesized that it plays a direct role in the conduction of an action potential along the axon; the possible relation between ACBM and this axonal receptor is of interest. In this paper we attempt to ascertain the function of ACBM by examining its interaction with some of the drugs and toxins which are known to block axonal conduction by specific mechanisms.

Experimental Section

Materials and Methods. The axon plasma membrane preparation was purified from the microsomal fraction of a hypotonic extract of the main sensory–motor nerve bundle from the eight walking legs of 1.5-lb lobsters, *Homarus americanus*. The details of this preparation and some of its characteristics have previously been described.[4]

The pharmacological agents tested and their sources are: *N*-acetylimidazole (Sigma); veratrine, mixture of alkaloids (Sigma); DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (Geigy); tetrodotoxin (Calbiochem); tetraethylammonium bromide (Eastman); procaine (Mann); hemicholinium 3 (Aldrich); phenobarbital (gift from Dr. C. Wilkinson); grayanotoxin I (gift from Dr. I. Yamamoto); and ouabain (Nutritional).

The binding of [^{3}H]nicotine (Amersham, specific activity 355 mCi/mmol) to the membrane preparation was measured by equilibrium dialysis. Aliquots of 0.35 ml containing 1–2 mg of protein/ml of the membrane preparation were dialyzed for 16 hr at 4° against 100 ml of lobster Ringers [457 m*M* NaCl, 15 m*M* KCl, 25 m*M* $CaCl_2 \cdot 2H_2O$, 4 m*M* $MgCl_2 \cdot 6H_2O$, 4 m*M* $MgSO_4 \cdot 3H_2O$, 10 m*M* Tris·HCl pH 7.5] containing the radioactive nicotine and the pharmacological agent being tested. At equilibrium, samples were taken of the contents of the dialysis bag and of the outer solution and their radioactivity was measured.[6] The difference represents the amount of [^{3}H]nicotine bound. In cases where some of the drugs showed no apparent effect on nicotine binding, they were also initially added directly at the desired concentration to the axon plasma membranes prior to dialysis. This control was to make sure that the membranes came in contact with the drug and was particularly relevant to the case of DDT which adsorbs strongly to the dialysis tubing and the glass walls of the flask. An additional factor that was necessary to consider under these conditions was the effect of the pharmacological agent on the equilibrium of [^{3}H]nicotine across the dialysis tubing. A control sample was run for each drug without axon plasma membranes. In all cases, after 16 hr identical concentrations of [^{3}H]nicotine were found inside and outside the dialysis bag.

The reversibility of the binding of those pharmacological agents which affected nicotine binding was tested by incubating the axon membranes with the compounds for 16 hr as in a regular equilibrium dialysis binding assay. After this time the dialysis bag containing the membrane and the drug was removed and placed in another flask containing 100 ml of [^{3}H]nicotine without the drug. The binding was measured in the usual manner after a second 16-hr equilibration time and compared with that of a control sample handled in an identical way but not exposed to the compound being tested. All of the compounds which inhibited nicotine binding were found to do so in a manner that was 93–98% reversible.

ATPases were assayed at 21° in 0.05 *M* Tris pH 7.5 with 3 m*M* $MgSO_4$ and 1 m*M* ATP in the presence or absence of 150 m*M* NaCl and 25 m*M* KCl. The reactions were stopped with 10% trichloroacetic acid and the inorganic phosphate released was measured by the method of Baginski, *et al.*[7] This technique has the advantage of not being sensitive to inorganic phosphate produced by acid hydrolysis of ATP after the color reagent has been added, because the excess molybdate is complexed by addition of a citrate arsenite solution.

Results

A summary of the interactions between the pharmacological agents and the ACBM is presented in Table I. The binding of [^{3}H]nicotine was used as a measure of the ACBM

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Table I. Effect of Pharmacological Agents on Binding of [^{3}H]Nicotine to the Axonal Cholinergic Binding Macromolecule

Compound	Type of inhibition	K_i, M	Physiological effect	Site of action
DDT (1×10^{-4})	0		Inhibits Na^+ inactivation and/or K^+ activation	
N-Acetylimidazole (1×10^{-4})	0			
Veratrin	Competitive	$3.6 \pm 0.3 \times 10^{-4}$ (2×10^{-4} g/ml)	K^+ activation	
Tetrodotoxin (2×10^{-6})	0		Inhibits Na^+ activation	Outside
TEA	Competitive	$1.6 \pm 0.1 \times 10^{-5}$	Inhibits K^+ activation	Inside
Procaine	Competitive	$2.9 \pm 0.2 \times 10^{-6}$	Inhibit Na^+ activation and K^+ activation	Inside
Hemicholinium 3	Competitive	$2.8 \pm 0.2 \times 10^{-5}$		
Phenobarbital (1×10^{-3})	0			
Grayanotoxin (5×10^{-4})	0		Increases Na^+ permeability	Either
Ouabain	Noncompetitive	$7.0 \pm 0.6 \times 10^{-5}$	Inhibits Na^+ pump	Outside

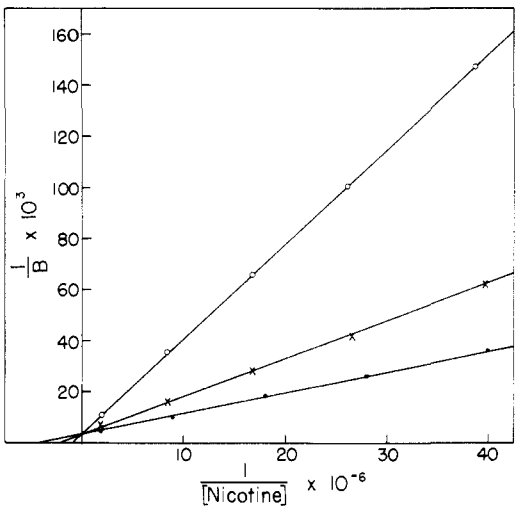

Figure 1. The effect of hemicholinium 3 on [^{3}H]nicotine binding. Double reciprocal plot of binding of nicotine, B (pmol/mg protein), to axon membranes in the absence of hemicholinium 3 (●–●–●), presence of 2.5×10^{-5} *M* hemicholinium 3 (×–×–×), and presence of 7.5×10^{-5} *M* hemicholinium 3 (○–○–○).

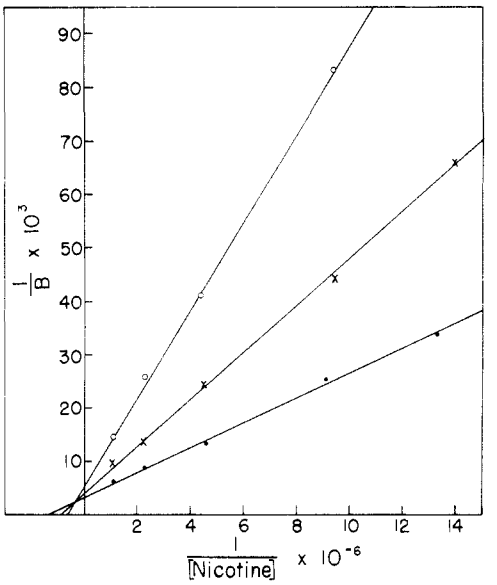

Figure 2. The effect of ouabain on [^{3}H]nicotine binding. Double reciprocal plot of binding of nicotine, B (pmol/mg protein), to axon membranes in the absence of ouabain (●–●–●), presence of 6×10^{-5} *M* ouabain (×–×–×), and presence of 2×10^{-4} *M* ouabain (○–○–○).

because it was previously shown that this cholinergic ligand was bound most tightly to the axon membranes ($K_D = 4 \times 10^{-7}$ *M*). The nature of the inhibition and the binding constants of the inhibitors (K_i) were determined from an examination of the binding of [^{3}H]nicotine in the concentration range of 10^{-8}–10^{-6} *M* both in the absence and in the presence of two different concentrations of inhibitor. Exemplary data are presented in the form of Lineweaver-Burk plots in Figures 1 and 2.

Tetrodotoxin specifically blocks the inward Na^+ current of the action potential[8] and interacts only with the external surface of the squid giant axon with an apparent dissociation constant of 10^{-9} *M*.[9] At concentrations of TTX as high as 2×10^{-6} *M*, no effect on nicotine binding was observed. These experiments were done within the range of pH of 6.0–6.5 because during the 16 hr required for the equilibrium dialysis binding assay the TTX is labile at the pH 7.8 which is usually used.

Tetraethylammonium specifically blocks the outward K^+ current of the action potential and interacts only with the internal surface of the squid axon.[10,11] It was observed to be a competitive inhibitor of nicotine binding with a $K_i = 1.6 \pm 0.1 \times 10^{-5}$ *M*.

Some compounds with properties related to the ability to block both inward Na^+ current and outward K^+ current of the action potential were also tested. Procaine, as previously reported,[4] was a competitive inhibitor of nicotine binding with a $K_i = 2.9 \pm 0.2 \times 10^{-6}$ *M* as was hemicholinium 3 (Figure 1) with a $K_i = 2.8 \pm 0.2 \times 10^{-5}$ *M*. Phenobarbital at concentrations up to 10^{-3} *M* had no effect on nicotine binding.

Compounds were studied which cause prolonged action potentials followed by repetitive firings of the axon. This is produced by preventing the inactivation of the inward Na^+ current and/or by blocking the outward K^+ current.[12,13] DDT at concentrations up to 10^{-4} *M* had no effect nor did *N*-acetylimidazole up to 10^{-4} *M*. Surprisingly, the mixture of veratrine alkaloids was found to be a competitive inhibitor of nicotine binding with a $K_i = 3.6 \pm 0.3 \times 10^{-4}$ *M* (2×10^{-4} g/ml). For this calculation an average molecular weight of 584 was used assuming that each of the components was present in equal amounts.

Grayanotoxin I, which increases the permeability of Na^+ of the resting axon membrane,‡ had no effect on nicotine binding at concentrations up to 10^{-4} *M*.

It was also surprising that ouabain, which blocks the energy-requiring Na^+ and K^+ fluxes of the resting membrane by inhibiting the Na^+K^+ATPase, also inhibited nicotine binding. As seen in Figure 2 ouabain was a noncompetitive inhibitor with a $K_i = 7.0 \pm 0.6 \times 10^{-5}$ *M*. This observation led to several experiments which attempted to examine the possibility that the ACBM is a component of the Na^+K^+ATPase.

Kinetic studies of the Na^+K^+ATPase were performed which

‡T. Narahashi, personal communication.

Table II. Effects of Salts on the Ouabain Inhibition of Nicotine Binding to Lobster ACBM Compared with the Binding of Ouabain to Electroplax of *Electrophorus electricus*

Assay conditions		% inhibition of binding of 10^{-7} *M* nicotine	[^{3}H]ouabain binding to electroplax (nmol/mg)[a]
0.05 *M* Tris pH 7.8		10	0.04
0.05 *M* Tris pH 7.8	+ 60 m*M* Na^+	29	
0.05 *M* Tris pH 7.8	+ 120 m*M* Na^+	23	
0.05 *M* Tris pH 7.8	+ lobster Ringers	45	
0.05 *M* Tris pH 7.8	+ 3 m*M* Mg^{2+}	15	0.20
0.05 *M* Tris pH 7.8	+ 120 m*M* Na^+ + 1 m*M* ATP	27	
0.05 *M* Tris pH 7.8	+ 3 m*M* Mg^{2+} + 1 m*M* ATP	19	0.28
0.05 *M* Tris pH 7.8	+ 120 m*M* Na^+ + 3 m*M* Mg^{2+} + 1 m*M* ATP	26	0.49

[a] See ref 18.

included three series of experiments. Within each series one of the three substrates, Na^+, K^+, and ATP, was varied over a nonsaturating range of concentrations while the other two were maintained at their optimal concentrations. In none of these experiments was any effect observed when nicotine at concentrations up to 10^{-5} *M* was included in the assay.

The binding of radioactive ouabain to membrane has been studied by several investigators and shown to be very sensitive to the ionic composition of the medium as well as to the presence of ATP.[14-18] Therefore, by using conditions which favor binding of ouabain to the Na^+K^+ATPase, one should expect to see a greater inhibition of nicotine binding in comparison to conditions less favorable for such binding. The results of these experiments are summarized in Table II. It was observed that whereas the presence of Na^+ enhanced the inhibition of 10^{-7} *M* nicotine binding caused by 6×10^{-5} *M* ouabain, it had a much larger effect on binding of ouabain to electroplax membranes. On the other hand, Mg^{2+} and ATP had no additional effect on the inhibition of nicotine binding while they greatly enhanced binding of ouabain. These results indicate that conditions which favor binding of ouabain to the Na^+K^+ATPase do not favor binding of ouabain to the site which is causing the noncompetitive inhibition of nicotine binding.

Discussion

We have previously suggested that ACBM is located on the internal surface of the axon plasma membrane and is a component common to both the Na^+ and K^+ gates.[4a] The suggestion was based on the following observations: the ACBM binds (1) local anesthetics which block both the inward Na^+ current and the outward K^+ current of the action potential and exert their effect on the internal surface of the axon plasma membrane;[19] (2) cholinergic agonists and antagonists which have specific effects on axonal conduction in lobster axons; (3) bungarotoxin which has no effect on axonal conduction yet can bind to the axon membrane vesicles *in vitro*.

The results presented here do not contradict this initial hypothesis although they do not help to assign definitively a physiological role for the ACBM. Some of the pharmacological agents tested did indeed block nicotine binding. Most of them did so in a competitive manner, and it is assumed that they were bound directly to the nicotine binding site. It was previously shown[4a] by mixed inhibitor studies that acetylcholine and procaine bound to the same site.

Another compound which has a local anesthetic effect when internally perfused in the squid giant axon, hemicholinium 3,[20] was also a competitive inhibitor of nicotine binding. Surprisingly, phenobarbital, which can also act as an anesthetic,[21] had no effect on nicotine binding. This may indicate that there is more than one mechanism for producing local anesthesia, assuming that one of these is caused by binding to the ACBM. This is further supported by the observation that the uncharged molecular form of the barbiturates is responsible for their blocking action, whereas with the basic local anesthetics it is the charged cationic form which is active on the inside of the membrane.[19] TEA which blocks the K^+ channel from the interior of the axon also blocked nicotine binding. These observations strengthen the hypothesis that the ACBM is on the interior surface of the axon plasma membrane, particularly since TTX which is active only when applied externally has no effect on nicotine binding.

The noncompetitive inhibition by ouabain, along with the observation that several of the pharmacological agents which blocked nicotine binding also inhibited ATPase,[3] suggested that the ACBM may be a component of the Na^+K^+ATPase. However, this possibility was eliminated by the fact that no interaction between nicotine (10^{-5} *M*) and the Na^+K^+ATPase could be detected by kinetic experiments, In addition, the ouabain inhibition was completely reversible and ionic conditions which favored (Na^+, Mg^{2+}, ATP) or inhibited (K^+) ouabain binding to the Na^+K^+ATPase of electroplax had no corresponding effect on the ouabain inhibition of nicotine binding to axons. This inhibition was probably caused by binding of ouabain to sites other than the Na^+K^+ATPase. Such "nonspecific" binding has been reported by several investigators.[14,16,17]

The major drawback in using pharmacological agents to define a physiological role for the ACBM is their lack of true specificity. For example, the physiological action of TEA on the axon is "specific" for the K^+ gate. However, biochemical studies show that TEA also interacts with acetylcholinesterase,[23] Na^+K^+ATPase,[3] and ACBM. Conversely, the fact that ACBM interacts with several pharmacological agents that are supposed to interact with the cation gates does not necessarily identify it with such a function.

A skeptical examination of the data presented here reveals that all of the drugs which block nicotine binding have a positively charged tertiary or quartenary nitrogen, with the exception of ouabain. This might suggest that any strongly anionic site in the membrane might have a strong affinity for nicotine and for these cationic agents. However, it must be remembered that this ACBM was initially characterized by its similarity to the postsynaptic cholinergic receptor (AChR) which exhibits a great amount of specificity in its binding abilities in spite of the fact that a large part of its energy of interaction with ligands comes from electrostatic attractions. There are important differences in the interactions of positively charged molecules with these two macromolecules. For example, ACBM has a much weaker affinity for acetylcholine ($K_D = 4.3 \times 10^{-5}$ *M*) in

comparison to AChR ($K_D = 8 \times 10^{-9}$ and $6.8 \times 10^{-8} M$). On the other hand, ACBM strongly binds TEA and procaine whereas these compounds at $10^{-4} M$ have no effect on binding of acetylcholine to AChR.§ Such differences imply that the interaction studied in this communication is indicative of a greater specificity than simple electrostatic attractions. In addition, it has recently been shown# that the affinity of the ACBM for nicotine is very dependent upon the ionic composition of the solution; the affinity increases as the concentration of NaCl is increased up to 2 *M*. This is the opposite of the effect one would expect if electrostatic attractions are the only determinants of the energies of interactions.

In conclusion, the evidence still indicates that the ACBM is on the internal surface of the axon plasma membrane and may be a component of both the Na^+ and K^+ gates. The independence of these two gates is shown pharmacologically by their differential responses to TTX and TEA. However, the interactions with local anesthetics still remain the strongest evidence that the ACBM may be a component common to both.

References

(1) B. Hille, *Progr. Biophys. Mol. Biol.*, **21**, 1 (1970).
(2) R. D. O'Brien, M. E. Eldefrawi, and A. T. Eldefrawi, *Annu. Rev. Pharmacol.*, **12**, 19 (1972).
(3) F. Matsumura and T. Narahashi, *Biochem. Pharmacol.*, **20**, 825 (1971).
(4) (a) J. L. Denburg, M. E. Eldefrawi, and R. D. O'Brien, *Proc. Nat. Acad. Sci. U. S.*, 69, 177 (1972); (b) J. L. Denburg, *Biochem. Biophys. Acta*, 282, 453 (1972).
(5) D. Nachmansohn, "Chemical and Molecular Basis of Nerve Activity," Academic Press, New York, N. Y., 1959.
(6) M. E. Eldefrawi, A. T. Eldefrawi, and R. D. O'Brien, *Mol. Pharmacol.*, 7, 104 (1971).
(7) E. S. Baginski, P. P. Foa, and B. Zak, *Clin. Chim. Acta*, 15, 155 (1967).
(8) T. Narahashi, J. W. Moore, and W. R. Scott, *J. Gen. Physiol.*, 47, 965 (1964).
(9) T. Narahashi, N. C. Anderson, and J. W. Moore, *ibid.*, **50**, 1413 (1967).
(10) I. Tasaki and S. Hagiwara, *ibid.*, **40**, 859 (1957).
(11) C. M. Armstrong, *ibid.*, **50**, 491 (1966).
(12) B. Hille, *ibid.*, 51, 199 (1968).
(13) T. Narahashi and H. G. Haas, *ibid.*, 51, 177 (1968).
(14) J. F. Hoffman, *ibid.*, **54**, 343 (1969).
(15) J. C. Ellory and R. D. Keynes, *Nature (London)*, 221, 776 (1969).
(16) P. F. Baker and J. S. Willis, *Biochim. Biophys. Acta*, 183, 646 (1969).
(17) D. Landowne and J. M. Ritchie, *J. Physiol.*, **207**, 529 (1970).
(18) R. W. Albers, G. J. Koval, and G. J. Siegel, *Mol. Pharmacol.*, 4, 324 (1968).
(19) T. Narahashi and D. T. Frazier, *Neurosci. Res.*, 4, 1 (1971).
(20) D. T. Frazier, T. Narahashi, and J. W. Moore, *Science*, 163, 820 (1969).
(21) P. Krupp, C. P. Bianchi, and G. Suarez-Kurtz, *J. Pharm. Pharmacol.*, 21, 763 (1969).
(22) T. Narahashi, D. T. Frazier, T. Deguchi, C. A. Cleaves, and M. C. Ernau, *J. Pharmacol. Exp. Ther.*, 177, 25 (1971).
(23) F. Iverson, *Mol. Pharmacol.*, 7, 129 (1971).

§M. Eldefrawi, unpublished results.
#J. S. Denburg, submitted for publication.

Synthesis of Methylene- and Carbonyl-Bridged Analogs of Iodothyronines and Iodothyroacetic Acids

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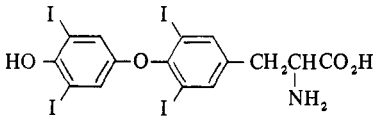
Syntheses are described of the methylene- and carbonyl-bridged analogs of a number of iodinated thyronines and thyroacetic acids. The critical synthetic step is arylation of 4-methoxybenzylidenetriphenylphosphorane with methyl 3,5-dinitro-4-chlorobenzoate. An ylide 11 is obtained which decomposes in methanol to yield methyl 3-(4-methoxyphenyl)-4-nitro-2,1-benzisoxazole-6-carboxylate (**13**). Selective reductions of **13** give methyl 3,5-diamino-4-(4-methoxybenzoyl)benzoate (**17**) and methyl 3,5-diamino-4-(4-methoxybenzyl)benzoate (**19**). These diamines are elaborated to the corresponding carbonyl- and methylene-bridged iodothyronines and iodothyroacetic acids.

Since the first synthesis of thyroxine[1] almost half a century ago, an impressive amount of effort has been directed toward the preparation of analogs of this deceptively simple structure (**1**). Much of such work has been stimulated by



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the observations that thyroxine-like activity can be retained, or even increased over that of the natural substance, by appropriate modification.[2] Phenolic-ring iodines can be replaced by alkyl groups, or the outer ring itself by 1-naphthyl.[3] In some instances, inner ring iodines can also be replaced by alkyl.[4] The alanine side chain can also be altered.[5]

A number of intriguing explanations have been advanced for the molecular action of thyroxine. It is known that thyroxine has a rigid, angulated structure, the phenolic ring being perpendicular and inclined at an angle of about 120° to the inner ring. There is also ample evidence to suggest that the outer ring of thyroxine undergoes oxidation, and that analogs which cannot be easily oxidized will not exhibit thyromimetic activity.[2,3a]

Inspection of space-filling models shows that, owing to the steric constraints imposed by the bulky iodine substituents, replacement of the diaryl ether linkage of thyroxine with CO or CH_2 changes the angulated structure very little. On the other hand, methylene substitution would be expected to increase the oxidation potential of the natural hormone considerably.[6] Carbonyl-bridged thyroxine would be even less readily oxidized. Thus, biological testing of these compounds should provide insights into the steric *vs.* electronic basis for the molecular action of thyroxine.

The replacement of the ether function of iodinated thyronines has received relatively little attention. Sulfur-,[7]