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Strychnine Binding in Rat Spinal Cord Membranes Associated with the Synaptic Glycine Receptor: Cooperativity of Glycine Interactions

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SUMMARY

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The saturable binding of [^{3}H]strychnine to synaptic membrane fractions of the rat spinal cord appears to involve an interaction with the synaptic receptor sites for the neurotransmitter actions of glycine. Binding exhibits a dissociation constant for strychnine of 2.6–4 nM and an EC_{50} for glycine inhibition of strychnine binding of 25 μM. Association kinetics is bimolecular, with a rate constant of 1.0×10^7 M^{-1} sec^{-1}, while dissociation of the strychnine-receptor complex is first-order, with a rate constant of 1.54×10^{-2} sec^{-1}. The dissociation constant (k_2/k_1) of 1.54 nM is similar to that obtained from equilibrium data. Monovalent cations increase specific and decrease nonspecific strychnine binding, while divalent cations are without effect. Detergents such as deoxycholate and Triton X-100 decrease receptor binding in concentrations which solubilize membrane protein. Displacement curves of [^{3}H]strychnine by glycine indicate cooperative interactions with a Hill coefficient of 1.7, whereas the Hill coefficient for displacement of [^{3}H]strychnine by unlabeled strychnine is 1.0. Diazonium tetrazole and acetic anhydride preferentially inhibit displacement of [^{3}H]strychnine binding by glycine and reduce the Hill coefficient of glycine displacement curves. Guanidine, *N*-ethylmaleimide, and increases in pH also lower the Hill coefficient for glycine displacement. Diazonium tetrazole slows the dissociation of [^{3}H]strychnine elicited by excess glycine without altering dissociation produced by excess unlabeled strychnine. Glycine and strychnine appear to bind to distinct sites which interact in a cooperative fashion.

INTRODUCTION

Glycine satisfies a variety of neurochemical (1–5) and neurophysiological (6–9) criteria as a major inhibitory neurotransmitter in the mammalian central nervous system, especially in the spinal cord and brain stem. The convulsant drug strychnine potently antagonizes natural inhibitory synaptic transmission in the spinal cord in a fashion similar to its antagonism of the inhibitory

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effects of iontophoretically applied glycine (10–13). Strychnine does not antagonize the effects of γ-aminobutyric acid. We have observed that radiolabeled strychnine of high specific activity binds to synaptic membrane fractions of mammalian spinal cord and brain stem in a specific manner, indicating an association with the synaptic glycine receptor (14, 15). The potencies of various amino acids for displacing specific strychnine binding parallels their glycine-like neurophysiological actions. The regional distribution of specific strychnine binding in the central nervous system closely correlates with that of endogenous glycine (16, 17), high-affinity synaptosomal glycine uptake (18–20), unique synaptosomal fractions accumulating glycine (21), and strychnine-sensitive synaptic inhibition (13, 22, 23).

Analysis of neurophysiological data for a number of neurotransmitter systems suggests that 2 or more transmitter molecules may be required to active one receptor (24–28). These data and the observation that glycine and strychnine bear no obvious structural resemblance to one another suggest that these two compounds may bind to the same receptor complex but at distinct sites, thus interacting with each other in a cooperative fashion (29–32). We describe here the properties of specific strychnine binding in synaptic membrane fractions of rat spinal cord, and the cooperative interaction of glycine with strychnine binding.

MATERIALS AND METHODS

Strychnine was obtained from Smith Kline & French and was labeled by catalytic tritium exchange at New England Nuclear Corporation and purified as described previously (14). The specific activity of the labeled strychnine was 13 Ci/mmole, as determined by comparison with the ultraviolet absorption of standard solutions at 254 nm.

Male Sprague-Dawley rats (100–150 g) were decapitated, and the spinal cords and brain stems were rapidly removed. The tissues were combined and homogenized in 20 volumes of ice-cold 0.32 M sucrose in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged for 10 min at 1000 × g, the pellet was discarded, and the resultant supernatant fluid was centrifuged for 20 min at 17,000 × g. The crude mitochondrial pellet was resuspended in 20 volumes of ice-cold distilled H_2O and homogenized with a Brinkmann Polytron PT-10 (setting 2.2) for 30 sec. The suspension was centrifuged for 20 min at 8000 × g. The supernatant fluid was collected, and the pellet, a bilayer with a soft buffy upper coat, was rinsed carefully with the supernatant fluid to collect the upper layer. The supernatant fluid was then centrifuged at 48,000 × g for 20 min. The final membrane pellets were stored at −30°. Specific strychnine binding was retained intact for at least 60 days under these storage conditions.

To measure specific binding of strychnine to spinal cord membranes, aliquots of crude synaptic membranes (0.3–1.0 mg of protein) were incubated in triplicate at 4° for 10 min in 2 ml of 0.05 M sodium-potassium phosphate buffer (pH 7.1) containing 200 mM NaCl and 2 nM [^{3}H]strychnine (38,000 cpm) alone or in the presence of 1 mM glycine, 0.1 mM strychnine, or various concentrations of drugs. After incubation, the reaction was terminated by centrifugation for 10 min at 48,000 × g. The supernatant fluid was decanted, and the pellet was rinsed with 5 and then 10 ml of ice-cold 0.15 M NaCl. Bound radioactivity was extracted into 1 ml of Protosol (New England Nuclear), 10 ml of toluene phosphor were added, and radioactivity was assayed by liquid scintillation spectrometry (Packard Tri-Carb model 3385 or 3375) at a counting efficiency of 34%. All binding determinations were conducted in triplicate, and for each determination parallel triplicate samples were assayed in the presence of 1 mM glycine and/or 0.1 mM strychnine to determine and correct for nonspecific strychnine binding. Except where specifically indicated, values for nonspecific binding were always the same whether strychnine or glycine was employed as a displacer.

For studies requiring rapid binding determinations, samples were filtered under pressure through Whatman glass fiber circles (GF-B) and washed once with 5 ml of cold 0.15 M NaCl (the filtering and washing required less than 8 sec). The filters were

shaken with 18 ml of Triton X-100 toluene phosphor for 20 min, and radioactivity was determined by liquid scintillation spectrometry. In parallel assays [^{3}H]strychnine binding values obtained by centrifugation and filtrations were the same. "Specific" [^{3}H]strychnine binding was obtained by subtracting from the total bound radioactivity the amount not displaced by high concentrations of glycine (1 mM) or strychnine (0.1 mM).

L-Cysteine, β-alanine, L-α-alanine, L-glutamic acid, L-isoleucine, L-methionine, L-serine, L-tryptophan, DL-threonine, and L-arginine were purchased from Calbiochem. L-Aspartic acid, γ-aminobutyric acid, glycine, L-lysine, L-tyrosine, L-leucine, L-proline, L-phenylalanine, and DL-β-aminoisobutyric acid were obtained from Sigma. Taurine was purchased from K & K Laboratories, and L-histidine was a product of Nutritional Biochemicals. Iodoacetate, iodoacetaamide, *N*-ethylmaleimide, 5,5′-dithiobis(2-nitrobenzoic acid), *p*-chloromercuribenzoic acid, 2,4-dinitrofluorobenzene, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, glycine O-methyl ester, 1-ethyl-3 - (3 - dimethylaminopropyl)carbodimide (33), tetranitromethane (34, 35), 2-hydroxy-5-nitrobenzyl (36, 37), and 2-methoxy-5-nitrobenzyl bromide (38) were all purchased from Sigma. Ethylenedinitrilotetraacetic acid was obtained from J. T. Baker Chemical Company, and diazonium tetrazole was made from 5-aminotetrazole (39, 40) purchased from Aldrich.

Proteins were measured according to Lowry *et al.* (41).

RESULTS

Saturation of specific [^{3}H]strychnine binding. The specific binding of [^{3}H]strychnine to synaptic membrane fractions of the spinal cord is a saturable process with respect to strychnine (Fig. 1). Specific binding is readily detected at 1 nM [^{3}H]strychnine and approaches saturation at 20 nM [^{3}H]strychnine. Half-maximal specific binding occurs at about 2.7 nM. By contrast, nonspecific binding, consisting of [^{3}H]strychnine binding in the presence of 1 mM glycine, is not saturable and increases linearly with increasing strychnine concentration. The ratio of specific to nonspecific binding decreases from values of about 5–6 at 1–4 nM to 3 at 20 nM strychnine. Routine assays employ 2 nM [^{3}H]strychnine.

Analysis of these equilibrium data by double-reciprocal and Scatchard plots indicated that a single high-affinity receptor site was being measured and that there is one population of independent binding sites. The maximum specific [^{3}H]strychnine binding capacity is about 1.8 pmoles/mg of crude synaptic membrane protein. The dissociation constant calculated from both these plots is 2.6–2.8 nM.

Nonradioactive strychnine displaces specific [^{3}H]strychnine binding in a saturable manner (Fig. 2). Half-maximal displacement occurs at about 4 nM, similar to the value for half-maximal saturation of [^{3}H]-strychnine. Displacement is essentially maximal at about 0.1 μM strychnine. The fact that half-maximal saturation occurs at the same concentrations of radioactive and nonradioactive strychnine indicates that the [^{3}H]strychnine is biologically equivalent to nonradioactive strychnine and that the calculated specific activity is valid. In addition, the similarity of values obtained in the present study, employing 200 mM sodium chloride in incubations, to those of a previous study omitting sodium chloride (14), indicates that the affinity of strychnine for the binding sites is the same in the presence and absence of sodium chloride.

Glycine also displaces [^{3}H]strychnine binding with half-maximal displacement at 25 μM and maximal displacement at 1 mM glycine. In routine assays "nonspecific binding" is determined by measuring [^{3}H]-strychnine binding in the presence of 1 mM glycine.

Kinetics of specific [^{3}H]strychnine binding. At 4°, specific [^{3}H]strychnine binding is a time-dependent process, attaining half-maximal binding at about 34 sec and reaching a plateau by 5 min (Fig. 3, lower). By contrast, nonspecific binding attains maximal values by 10 sec, the first time interval examined. These data satisfy models for a bimolecular reaction with a rate constant for

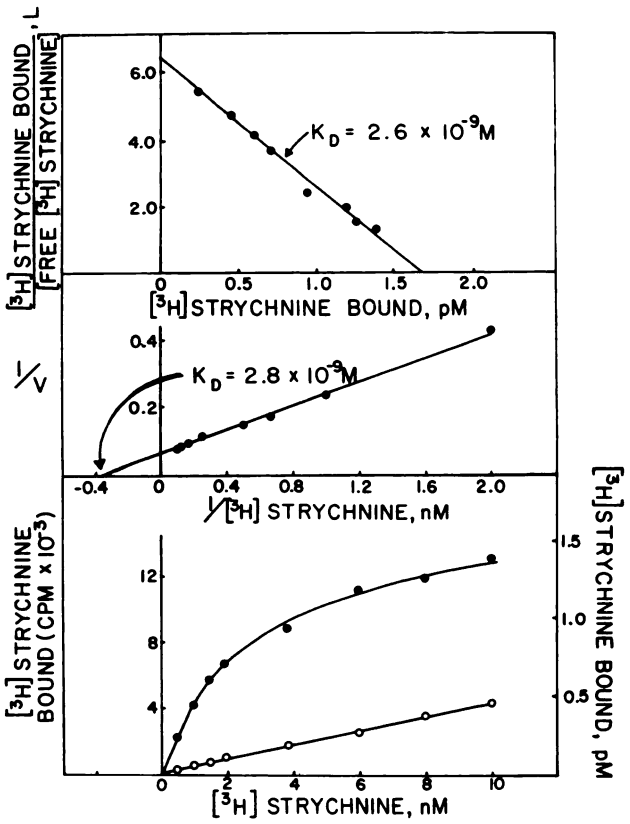

FIG. 1. *Saturation of specific strychnine binding with increasing concentrations of [³H]strychnine*
Synaptic membrane suspensions (1.0 mg of protein per tube) were incubated with increasing concentrations of [³H]strychnine at 4° for 10 min. Nonspecific binding (○) was obtained from the binding in the presence of 1 mM glycine. Specific binding (●) was obtained after subtraction of nonspecific from total binding. Bottom, data plotted on linear axes; center, data represented as a double-reciprocal analysis; top, data plotted according to the method of Scatchard. Values are the means of triplicate determinations. The experiment was replicated three times.

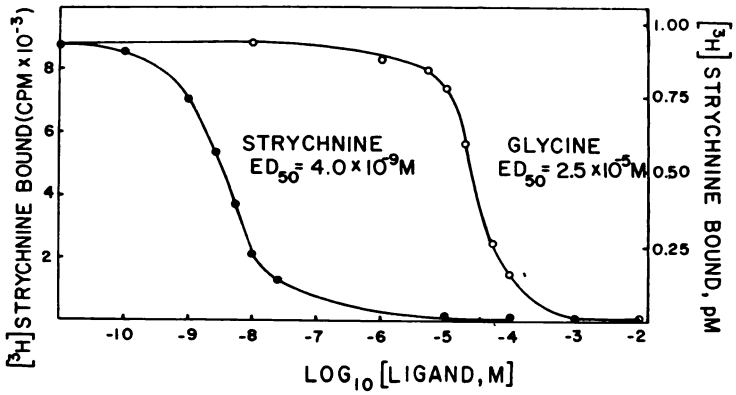

FIG. 2. *Displacement of [³H]strychnine binding by nonradioactive glycine and nonradioactive strychnine*
Synaptic membrane suspensions (1.0 mg of protein per tube) were incubated with 2 nM [³H]strychnine (38,000 cpm) and increasing amounts of glycine or strychnine at 4° for 10 min. Nonspecific binding obtained in the presence of 10 mM glycine or 0.1 mM strychnine has been subtracted from all experimental points. Values are the means of triplicate determinations, which varied less than 15%. The experiment was replicated four times.

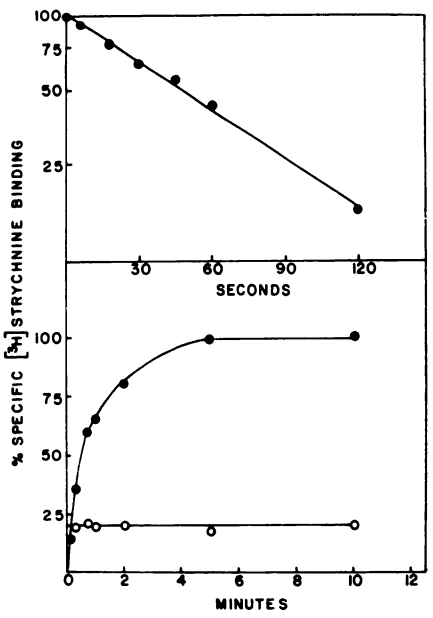

FIG. 3. *Rates of association and dissociation of [³H]strychnine binding to rat brain stem–spinal cord synaptic membranes*

Upper, semilogarithmic plot of the dissociation of bound [³H]strychnine as a function of time at 4°. Suspensions of synaptic membranes (0.4 mg of protein per assay tube) were incubated for 10 min at 4° with 2 nM [³H]strychnine (38,000 cpm) as in the standard assay described in the text. Nonradioactive glycine (1 mM) was then added rapidly. Samples were stirred and filtered immediately (zero time) on Whatman glass fiber circles or were incubated for longer periods before they were filtered and washed with 5 ml of ice-cold 0.15 M NaCl. Points represent the means of triplicate determinations, which varied less than 10%. Values have been corrected for nonspecific binding and are given as percentages of control binding obtained at zero time (0.7 pmole of [³H]strychnine bound per milligram of membrane protein). Lower, rate of binding at 4° of 2 nM [³H]strychnine to synaptic membranes (0.4 mg of protein per assay tube). The incubation was terminated by filtration on Whatman glass fiber circles. Radioactivity bound to the filter paper in the absence of tissue (800 cpm) has been subtracted from all experimental points. The nonspecific binding is the binding obtained in the presence of 1.0 mM glycine. Points represent the means of triplicate determinations, which varied less than 10%. Values are given as percentages of control binding at 10 min (0.7 pmole of [³H]strychnine bound per milligram of membrane protein). ●, specific [³H]strychnine binding; ○, nonspecific [³H]strychnine binding.

strychnine-receptor association, k_1, of about $1.0 \pm 0.1 \times 10^7$ M^{-1} sec^{-1}, which is similar to the value attained in a previous study in which sodium chloride was not included in the incubation medium (14).

The rate of dissociation of the strychnine-receptor complex cannot be measured readily at 15° or 25°, because under these conditions dissociation appears to be completed by 20 sec. At 4° we examined the rate of dissociation using 1 mM glycine to displace [³H]strychnine (Fig. 3, upper). Dissociation of the strychnine-receptor complex appears to be a first-order process. When plotted semilogarithmically, the half-life of the strychnine-receptor complex is about 45 sec, or 50% greater than the 30-sec value obtained in experiments without sodium in the incubation medium (14). However, because of the great rapidity of dissociation, we cannot be certain whether the difference between the rates of dissociation under these two different conditions is meaningful. The rate constant for dissociation at 4°, k_2, is 1.54×10^{-2} sec^{-1}. The dissociation constant (k_2/k_1) is 1.54×10^{-9} M.

Competition of amino acids for specific [³H]strychnine binding. In a previous study we observed that the potency of a variety of amino acids in displacing specific [³H] strychnine binding closely parallels their ability to mimic the neurophysiological actions of glycine (14). Glycine and β-alanine, the most potent neurophysiologically, were also the most potent amino acids in displacing [³H]strychnine binding, while taurine, DL-β-aminoisobutyric acid, and L-α-alanine were second most potent both in glycine-like neurophysiological activity and in displacing bound strychnine. In the present study we have examined these as well as a variety of other amino acids to determine the specificity of [³H] strychnine binding (Table 1). Under the present assay conditions, which include Na^+, as in the previous study, which omitted sodium chloride from the incubation, β-alanine and taurine maximally displace [³H]strychnine binding to the same extent as glycine. At 10 μM, displacement by β-alanine is the same as by glycine, while taurine is somewhat less effective. At 1 mM,

TABLE 1

Amino acid competition for specific [³H]strychnine binding

Specific [³H]strychnine binding to crude synaptic membrane suspensions (0.35 mg of membrane protein) was determined as described in the text in the presence and absence of various amino acids in the indicated concentrations. Values are given as percentage displacement of specific [³H]strychnine binding by 1 mM glycine (0.6 pmol/mg of membrane protein) and represent the means of three determinations, which varied less than 10%.

Amino acid	Binding at		
	10^{-5} M	10^{-4} M	10^{-3} M
	%	%	%
α-Amino acids			
Glycine	16	85	100
L-α-alanine	0	20	71
Proline			63
L-serine	0	13	47
Tryptophan			47
Isoleucine			32
Phenylalanine			31
Cysteine			29
Valine			18
Histidine			11
Methionine			11
Threonine			9
Lysine			0
Arginine			0
Leucine			0
Glutamic acid			0
Aspartic acid			0
β-Amino acids			
β-Alanine	16	89	103
DL-β-Aminoisobutyric acid	0	22	81
Taurine	9	50	104
γ-Amino acids			
γ-Aminobutyric acid	0	0	15

L-α-alanine and L-proline displace specific strychnine binding about 60–70% while cysteine, isoleucine, phenylalanine, and tryptophan displace binding only about 25–50%. Several other amino acids with no effect on specific binding include methionine, glutamic and aspartic acids, arginine, leucine, valine, lysine, histidine, threonine, and γ-aminobutyric acid.

Influence of cations on specific [³H]strychnine binding. Sodium chloride increases specific and decreases nonspecific [³H]strychnine binding (Table 2). At 50 mM NaCl, nonspecific binding is decreased by 30%, and a maximal decline in nonspecific binding of 40% is detected at 300 mM NaCl, after which nonspecific binding gradually increases with further increments of NaCl. No change in specific binding, i.e., glycine-displaceable strychnine binding, occurs until 200 mM NaCl, when specific binding is enhanced 30%. Specific [³H]strychnine binding increases progressively with additional NaCl to a maximum of 200% at 1.6 M NaCl, and then falls somewhat at 2.0 M NaCl. Because of the simultaneous increase in specific and decrease in nonspecific binding, the ratio of specific to nonspecific [³H]strychnine binding increases from a value of about 2 in the absence of sodium to about 5.2 at 1 M NaCl. This ratio then falls to 4.4 and 2.7 at 1.6 and 2.0 M NaCl, respectively. Our routine binding incubation mixtures contain 0.2 M NaCl.

KCl and LiCl affect binding in a fashion similar to NaCl, increasing specific and decreasing nonspecific binding. With both KCl and LiCl, a 30% enhancement of specific binding is detected at 0.1 M; a 60–75% increase is observed at 0.5 M, the concentration at which the ratio of specific to nonspecific binding is increased up to about 5.0.

In contrast to the enhancing effect of monovalent cations, divalent cations have little influence on specific strychnine binding (Table 2). Calcium, magnesium, and barium ions at 5 or 50 mM fail to alter binding. Moreover, the chelating agents EDTA and ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, in concentrations of 5–100 mM, do not alter binding.

Thermal stability and pH dependence of specific [³H]strychnine binding. Specific [³H]strychnine binding occurs optimally over a broad pH range of 6.0–8.0 with citric acid, sodium-potassium phosphate, and Tris buffers (Fig. 4). At more acid pH values specific binding decreases, so that at pH 5.0 binding is half the maximal values. At more alkaline pH values binding decreases to values at pH 9 only 25–30% of the maximal values obtained at neutral pH.

TABLE 2

Effects of EDTA, ethylene glycol bis(β-aminoethyl(ether)-N,N'-tetraacetic acid (EGTA), and various ions on specific [³H]strychnine binding

Specific [³H]strychnine binding to crude synaptic membrane suspensions (0.6 mg of membrane protein) was determined as described in the text, except that 200 mM sodium chloride was excluded from the assay and various salts and chelating agents were added to the assay mixtures as indicated. Specific [³H]strychnine binding for controls in the absence of salt was 0.4 pmole/mg of membrane protein. Values are expressed as a percentage of controls and represent the means of three determinations, which varied less than 10%.

Agent	Specific [³H]strychnine binding	Specific to nonspecific binding ratio	Agent	Specific [³H]strychnine binding	Specific to nonspecific binding ratio
	% control			*% control*	
None	100	2.0	$CaCl_2$, 5 mM;		
NaCl, 50 mM	100	3.0	NaCl, 200 mM	103	
NaCl, 100 mM	97	3.0	$CaCl_2$, 50 mM;		
NaCl, 150 mM	109	3.5	NaCl, 200 mM	103	
NaCl, 200 mM	130	4.0	$BaCl_2$, 5 mM;		
NaCl, 400 mM	143	5.0	NaCl, 200 mM	99	
NaCl, 1.0 M	171	5.2	$BaCl_2$, 50 mM;		
NaCl, 1.6 M	212	4.4	NaCl, 200 mM	102	
NaCl, 2.0 M	186	2.7	EDTA, 5 mM;		
KCl, 100 mM	130	3.5	NaCl, 200 mM	104	
KCl, 200 mM	159	3.9	EDTA, 10 mM;		
KCl, 500 mM	176	5.3	NaCl, 200 mM	104	
LiCl, 100 mM	130	3.5	EDTA, 100 mM;		
LiCl, 200 mM	163	4.2	NaCl, 200 mM	108	
LiCl, 500 mM	158	4.6	EGTA, 5 mM;		
$MgCl_2$, 5 mM;			NaCl, 200 mM	99	
NaCl, 200 mM	103		EDTA, 10 mM;		
$MgCl_2$, 50 mM;			NaCl, 200 mM	97	
NaCl, 200 mM	104		EGTA, 100 mM;		
			NaCl, 200 mM	90	

The thermal stability of specific strychnine binding was examined by incubating synaptic membrane fractions at various temperatures for 10 min prior to the standard binding assay. Binding is unaffected by treatment at temperatures up to 55° but then falls precipitously and is abolished by heating for 10 min at 70°.

Influence of detergents and denaturing agents on specific [³H]strychnine binding. Triton X-100 progressively lowers strychnine binding (Table 3). At 0.01%, Triton produces a 5% increase in specific binding, a finding obtained consistently in repeated experiments. In previous studies omitting NaCl from the incubation, Triton increased specific binding about 25% and was included in routine assays (14). At higher concentrations, Triton progressively reduces specific binding. At 1.0% Triton, specific binding is lowered to about one-third of values obtained in the absence of Triton. Nonspecific binding is also lowered by Triton, with about a 70% maximal reduction at 2% Triton. When the exposure to Triton is lengthened from 15 min to 60 min, specific binding is reduced an additional 10–20% and the augmenting effect of 0.01% Triton on binding is no longer observed.

Treatment with deoxycholate for 15 min reduces binding similarly to treatment with comparable concentrations of Triton (Table 3). Thus 0.2% concentrations of both detergents reduce binding about 50%. At 0.4%, deoxycholate reduces binding by 73%, while 0.5% Triton reduces binding about 50%.

Both urea and guanidine HCl, which disrupt the tertiary structure of proteins, markedly disrupt [³H]strychnine binding

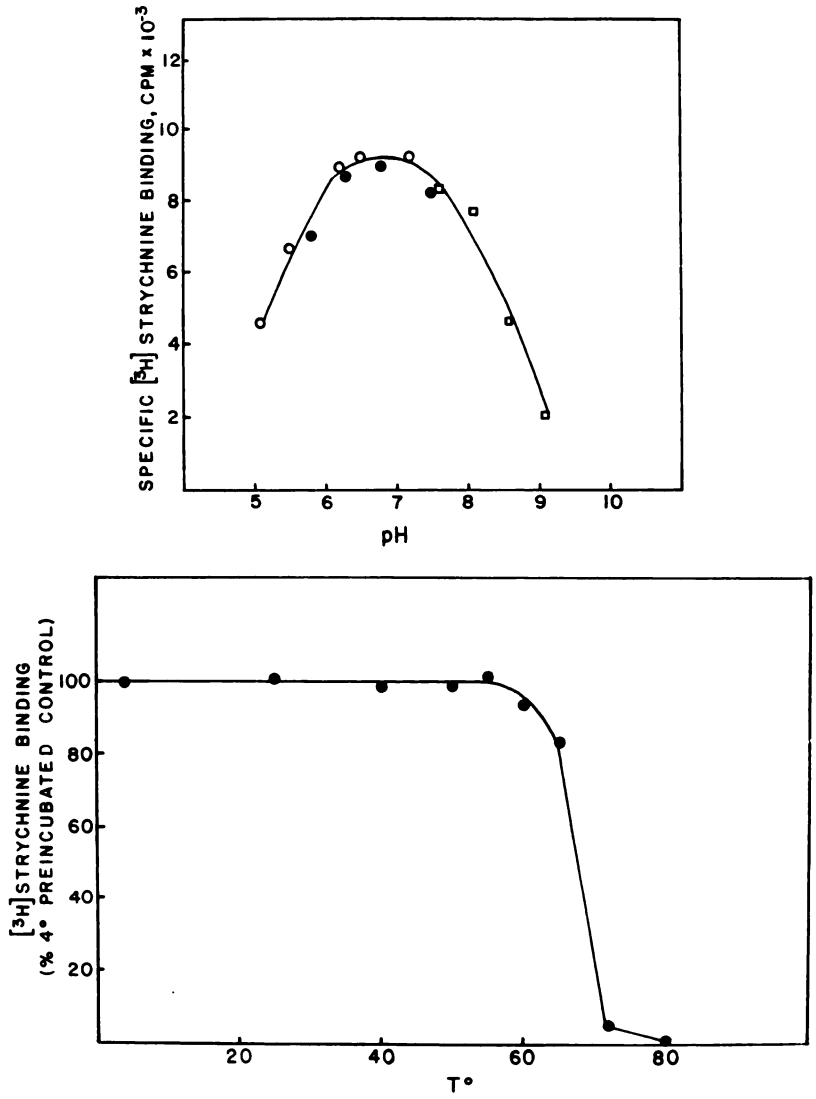

FIG. 4. *Effects of temperature and pH on specific binding of [³H]strychnine to brain stem-spinal cord synaptic membranes*

Upper, suspensions of crude synaptic membranes (1.0 mg of protein per assay tube) were added to tubes containing buffers at the pH indicated, and specific [³H]strychnine binding was determined as described in the text. The buffers used were 0.05 M sodium citrate (○), 0.05 M sodium phosphate (●), and 0.05 M Tris (□). Lower, suspensions of crude synaptic membranes (0.5 mg of protein per assay tube) in 0.1 M phosphate buffer, pH 7.1, were kept in an ice bath. Samples were then incubated at the indicated temperatures for 10 min and returned to the ice bath. Then 1 ml of 0.4 M NaCl was added, and the specific binding of [³H]strychnine was determined as described in the text. Values are given as percentages of control binding obtained after a 10-min preliminary incubation at 4° (0.65 pmole of [³H]strychnine bound per milligram of membrane protein).

(Table 4). Binding is very sensitive to guanidine, with 50% inhibition at 10 mM concentrations and 96% inhibition at 100 mM. Urea produces 70% inhibition at 1 M and complete inhibition at 2 M. These concentrations of guanidine and probably also of urea are not high enough to alter profoundly the quaternary or tertiary struc-

TABLE 3

Effect of detergents on specific [³H]strychnine binding in synaptic membrane fractions

Detergents were added as indicated to suspensions of crude synaptic membranes (0.4 mg of protein) in 0.05 M sodium phosphate buffer, pH 7.1, containing 200 mM sodium chloride, and incubated at 4° for the indicated times. Specific [³H]strychnine binding was then determined as described in the text. The specific [³H]strychnine binding for controls in the absence of detergent was 0.65 pmole/mg of membrane protein. Values are given as percentages of controls and represent the means of three determinations, which varied less than 10%.

Detergent	Concentration[a]	Specific [³H]strychnine binding	
		15 min	60 min
	%	% control	
Triton X-100	0.010	105	81
	0.025	92	80
	0.125	59	50
	0.250	52	44
	0.50	50	31
	1.00	38	28
Sodium deoxycholate	0.01		93
	0.20		43
	0.40		27

[a] Triton X-100 concentrations are volume for volume; deoxycholate concentrations are weight per volume.

TABLE 4

Effects of urea and guanidine HCl on specific [³H]strychnine binding, and reversibility of these effects

Suspensions of crude synaptic membranes (0.35 mg of protein) in 2 ml of 0.05 M sodium phosphate buffer, pH 7.1, containing 200 mM sodium chloride and the indicated reagent, were incubated at 4° for 30 min. Specific [³H]strychnine binding was then determined as described in the text. For reversibility determinations, samples treated as described above were diluted with 10 ml of 0.15 M NaCl and centrifuged for 15 min at 48,000 × *g*. The pellets were resuspended in an additional 10 ml of 0.9% NaCl and centrifuged again for 15 min at 48,000 × *g*. The final pellets were then resuspended in 2 ml of 0.05 M sodium phosphate buffer, pH 7.1, containing 200 mM sodium chloride, and assayed for specific [³H]strychnine binding as described in the text. The specific [³H]strychnine binding for controls was 0.6 pmole/mg of membrane protein. Values are given as percentages of controls and represent the means of three determinations, which varied less than 10%.

Reagent	Specific [³H]strychnine binding	
	Before removal	After removal
	% control	
Urea, 1 M	30	100
Urea, 2 M	8	100
Urea, 3 M	0	100
Urea, 5 M	0	85
Guanidine HCl, 1 mM	95	100
Guanidine HCl, 10 mM	54	100
Guanidine HCl, 100 mM	4	100
Guanidine HCl, 1 M	0	70
Guanidine HCl, 2 M	0	36
Guanidine HCl, 4 M	0	7

ture of protein, and the significance of their effects is not clear. The effects of guanidine and urea are reversible up to 1 M and 5 M, respectively, after which there is apparent irreversible denaturation.

Effect of membrane protein-modifying reagents on specific [³H]strychnine binding. Several chemical reagents which modify protein residues were tested for their influence on specific [³H]strychnine binding (Table 5). In 0.1 mM concentrations iodoacetate, 5,5′-dithiobis(2-nitrobenzoic acid), and *p*-chloromercuribenzoate do not alter strychnine binding. Iodoacetamide and *N*-ethylmaleimide have no significant effect up to 5 and 4 mM, respectively. The reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, which reacts primarily with protein carboxyl groups (33), does not significantly affect either total or specific strychnine binding in the presence or absence of glycine *O*-methyl ester. The tryptophan-modifying reagents 2-hydroxy-5-nitrobenzyl bromide (36, 37) and 2-methoxy-5-nitrobenzyl bromide (38) have different effects. Specific strychnine binding is lowered 65% by 2-hydroxy-5-nitrobenzyl bromide (4 mM), but the reagent markedly increases total strychnine binding and nonspecific strychnine binding. These results are difficult to interpret, because the apparent inhibi-

TABLE 5

Effect of prior treatment of spinal cord membranes with various protein reagents on total and specific [^3H]strychnine binding

Various reagents, dissolved in the indicated solvents, were added to aliquots of crude synaptic membranes (1.0 mg of protein per milliliter of 0.1 M sodium phosphate buffer, pH 7.1), and the reactions were allowed to proceed for 1 hr at 4°. Then 10 ml of 0.15 M NaCl were added, and the suspensions were centrifuged for 15 min at 48,000 × *g*. The pellets were resuspended in 2 ml of 0.05 M sodium phosphate buffer, pH 7.1, and 200 mM sodium chloride, and [^{3}H]strychnine binding was assayed as described in the text. The specific [^{3}H]strychnine binding for controls was 0.7 pmole/mg of membrane protein. Values are given as percentages of controls and represent the means of three determinations, which varied less than 10%.

Solvent and reagent	Total [^{3}H]strychnine binding	"Specific" [^{3}H]strychnine binding
		% control
Phosphate[a]		
Diazonium tetrazole, 2.5 mM	85	0
Diazonium tetrazole, 1.25 mM	90	18
Diazonium tetrazole, 0.75 mM	95	70
Diazonium tetrazole, 0.25 mM	100	90
Diazonium tetrazole, 0.025 mM	100	100
Ethanol[b]		
Acetic anhydride, 50 mM	70	10
Acetic anhydride, 25 mM	98	30
Acetic anhydride, 10 mM	100	69
Acetic anhydride, 1 mM	100	93
Acetic anhydride, 50 mM	100	100
2,4-Dinitrofluorobenzene, 4 mM	37	26
Tetranitromethane, 5 mM	52	40
Acetone[b]		
2-Hydroxy-5-nitrobenzyl bromide, 4 mM	150	39
2-Methoxy-5-nitrobenzyl bromide, 4 mM	96	85
Water		
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, 20 mM	92	89
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, 20 mM, + glycine *O*-methyl ester, 50 mM	98	97
Iodoacetate, 0.1 mM	100	100
Iodoacetamide, 5 mM	96	95
N-Ethylmaleimide, 0.1 mM	100	100
N-Ethylmaleimide, 4.0 mM	93	91
5,5′-Dithiobis(2-nitrobenzoic acid), 0.1 mM	100	100
p-Chloromercuribenzoate, 0.1 mM	100	100

[a] Sodium phosphate buffer, 0.1 M, pH 7.1.

[b] The final concentration of organic solvent was less than 1% of the total volume of the incubation mixture. This concentration of ethanol and acetone does not affect specific binding of [^{3}H]strychnine under these conditions.

tion of specific strychnine binding may result from decreased resolution due to increased nonspecific binding. Treatment with 2-methoxy-5-nitrobenzyl bromide (4 mM) does not alter either specific or nonspecific binding.

Tetranitromethane (5 mM), a reagent that modifies tyrosyl (34) and perhaps tryptophanyl residues (35) and oxidizes sulfhydryl groups, inhibits both total and specific strychnine binding 50–60%. Dinitrofluorobenzene (4 mM), which modifies many functional groups, also decreases both total and specific binding (65–75%).

Diazonium tetrazole, which is relatively specific for histidyl and tyrosyl residues (39) but which also reacts with tryptophanyl groups (40), completely abolishes specific strychnine binding at 2.5 mM but has little effect on total strychnine binding. Simi-

larly, acetic anhydride, which reacts with amino groups and tyrosyl residues, also inhibits specific and total strychnine binding differentially, having a more marked effect on specific strychnine binding than on total strychnine binding. These results indicate that the modifications produced by these reagents interfere with the displacement of strychnine by glycine without altering total strychnine binding. A possible explanation is that glycine displaces strychnine by acting at a site other than the strychnine binding site.

Differential effects of protein reagents on displacement by glycine or strychnine of [^{3}H] strychnine binding. Experiments were performed to compare the influence of membrane treatment with protein-modifying reagents on the displacement of [^{3}H]strychnine binding by nonradioactive strychnine or glycine (Table 6). Both diazonium tetrazole and acetic anhydride affect displacement by strychnine and glycine differentially. Thus 2.5 mM diazonium tetrazole does not significantly alter the total [^{3}H] strychnine bound but completely prevents the ability of 1 mM glycine to displace [^{3}]strychnine. In contrast, displacement by nonradioactive strychnine is reduced only 50% in the same preparations. Acetic anhydride lowers total [^{3}H]strychnine binding about 30%, yet decreases the ability of glycine to displace [^{3}H]strychnine binding 90% while lowering the displacement by nonradioactive strychnine only 50%.

In contrast to the effects of diazonium tetrazole and acetic anhydride, 2,4-dinitrofluorobenzene, tetranitromethane, and *N*-ethylmaleimide lower the total amount of [^{3}H]strychnine bound but do not affect the ability of either strychnine or glycine to displace [^{3}H]strychnine binding. Dinitrofluorobenzene (4 mM) and tetranitromethane (5 mM) reduce specific [^{3}H]strychnine binding about 70% and 60%, respectively, while *N*-ethylmaleimide (4 mM) decreases binding only 8%.

To demonstrate further the differential effects of diazonium tetrazole on glycine or strychnine displacement of [^{3}H]strychnine binding, protection experiments were performed (Table 7). Glycine at a final concentration of 0.1 mM is able to provide partial protection against the reagent. Higher concentrations yield full protection from the effects of diazonium tetrazole. γ-Aminobutyric acid also protects but less effectively than glycine, presumably because of its lower affinity for the glycine binding site. Histidine at 1 mM also renders the dia-

TABLE 6

Effects of protein-modifying reagents on specific and nonspecific [^{3}H]strychnine binding

Suspensions of crude synaptic membranes (1.0 mg of protein per milliliter of 0.1 M sodium phosphate buffer, pH 7.1) were treated with the indicated reagents as described in Table 5. The membranes were then assayed for [^{3}H]strychnine binding, using both excess glycine (1 mM) and nonradioactive strychnine (0.1 mM) to determine "nonspecific" binding. Values represent the means of triplicate determinations, which varied less than 15%. The experiment was replicated three times.

Reagent	Concentration	[^{3}H]Strychnine (A)	[^{3}H]Strychnine + 0.1 mM unlabeled strychnine (B)	Δ [(A − B)/8300]	[^{3}H]Strychnine + 1 mM unlabeled glycine (C)	Δ [(A − C)/8100]
	mM	*cpm/mg protein*	*cpm/mg protein*	*% control*	*cpm/mg protein*	*% control*
Control	2.5	9600	1300	100	1500	100
Diazonium tetrazole	2.5	8160	3927	51	8200	0
	1.25	8640	3660	60	7182	18
	0.75	9120	1430	93	3450	70
	0.25	9580	1400	98	2290	90
Acetic anhydride	50	6686	2407	51	5904	10
	25	9490	2500	84	7134	29
	10	9610	1490	98	4021	69
2,4-Dinitrofluorobenzene	4.0	3632	1277	28	1485	27
Tetranitromethane	5	4977	1461	42	1699	40
N-Ethylmaleimide	4	9000	1350	92	1620	91

TABLE 7

Effects of membrane treatment with diazonium tetrazole in the presence of various concentrations of strychnine, glycine, γ-aminobutyric acid, and histidine on specific [³H]strychnine binding

Suspensions of crude synaptic membranes (0.9 mg of membrane protein per milliliter of 0.1 M sodium potassium phosphate buffer, pH 7.1) were treated with diazonium tetrazole as described in Table 6, in either the presence or absence of various concentrations of nonradioactive strychnine, glycine, γ-aminobutyric acid, or histidine. The membranes were washed with 100 volumes of ice-cold 0.9% NaCl three times, then assayed for strychnine binding. Specific [³H]-strychnine binding for controls was 0.6 pmole (5650 cpm)/mg of membrane protein. Values are given as percentages of controls, and the experiment was replicated three times.

Protection reagent	Concentration	Diazonium tetrazole	Strychnine-displaceable [³H]strychnine binding	Glycine-displaceable [³H]strychnine binding
	mM	*mM*	*% control*	*% control*
None			100	100
		1.0	85	33
Glycine	0.2	1.0	103	46
	0.5	1.0	92	68
	1.0	1.0	94	81
γ-Aminobutyric acid	0.2	1.0	88	35
	0.5	1.0	89	35
	1.0	1.0	98	65
Histidine	0.2	1.0	90	67
	1.0	1.0	90	93
Strychnine	0.1	1.0	71	2
	1.0	1.0	64	0

zonium tetrazole ineffective, probably by reacting directly with the reagent (39).

By contrast, strychnine even at 1 mM affords no protection of the membranes from diazonium tetrazole treatment. In fact, it seems to enhance slightly the ability of the reagent to inhibit glycine displacement selectively. These data further support the suggestion that glycine and strychnine bind to separate but mutually interacting sites.

Displacement of [³H]strychnine by varying concentrations of glycine and strychnine. The differential effects of diazonium tetrazole and acetic anhydride on the ability of glycine and strychnine to displace [³H]strychnine binding suggest that glycine and strychnine may bind to two separate but mutually interacting binding sites. To examine this possibility, we performed the following experiments.

In one study we examined the saturation of [³H]strychnine binding in the presence of two concentrations of either nonradioactive strychnine or glycine (Fig. 5). Double-reciprocal analysis indicates that nonradioactive strychnine displaces [³H]strychnine in a strictly competitive fashion. By contrast, similar plots for glycine are nonlinear and appear concave upward, similar to patterns with some allosteric enzymes (42–45).

Hill plots for the displacement of [³H] strychnine by nonradioactive strychnine or glycine are linear, with an n for strychnine of 1.0 ± 0.1, while the n for glycine is 1.7 ± 0.08 (Fig. 6). This indicates that glycine displaces [³H]strychnine binding in a cooperative fashion.

Effects of diazonium tetrazole and acetic anhydride on [³H]strychnine displacement by varying strychnine and glycine concentrations. The preferential influence of diazonium tetrazole on glycine displacement of strychnine suggests that diazonium tetrazole interferes with the cooperative interaction of glycine with strychnine binding sites. To examine this possibility, Hill plots were obtained for the displacement of [³H]strychnine binding by glycine in synaptic membranes treated with low concentrations of diazonium tetrazole, which reduce specific strychnine binding less than 30% (Fig. 7). As observed previously, the Hill coefficient for displacement by glycine in the absence of drug treatment is 1.7. Diazonium tetrazole (0.25 mM) reduces the Hill coefficient to 1.2, and at 0.75 mM diazonium tetrazole reduces it to 1.1. These data indicate that diazonium tetrazole may interfere with the cooperative interaction of glycine with strychnine binding sites.

Acetic anhydride-treated membranes similarly display an apparent decrease in cooperativity between glycine and strychnine binding sites. The Hill coefficient is de-

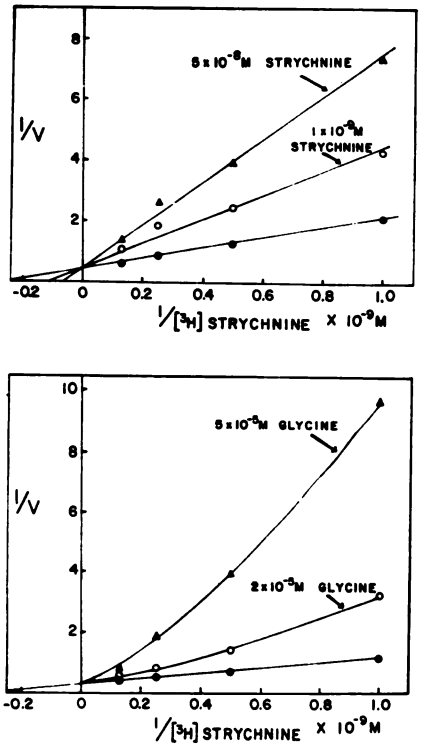

FIG. 5. *Competition of nonradioactive glycine or strychnine with [³H]strychnine in synaptic membrane binding assays*

Synaptic membrane suspensions (1.0 mg of protein per tube) were incubated with increasing amounts of [³H]strychnine and in the absence or presence of nonradioactive glycine (lower) or nonradioactive strychnine (upper). Nonspecific binding obtained in the presence of 1 mM glycine or 0.1 mM strychnine has been subtracted from all experimental values. Data were plotted by double-reciprocal analysis. "Velocity" (V) represents specific [³H]strychnine binding (counts per minute per milligram of membrane protein, uncorrected for 34% counting efficiency) at the various [³H]-strychnine concentrations indicated. Values are the means of triplicate determinations, which varied less than 15%. The experiment was repeated twice.

creased from 1.7 with control membranes to 1.2 after treatment with 10 mM acetic anhydride (Fig. 8).

Diazonium tetrazole could interfere with the glycine cooperativity either by altering the glycine site itself, by disrupting cooperative interactions of the glycine and strychnine sites, or by a combination of these two actions. If diazonium tetrazole exerts its effects solely by inactivating a proportion of glycine sites on the receptor, then glycine interactions with the remaining sites should be normal. In this case the dissociation rate of [³H]strychnine measured in the presence of excess glycine would be unaffected by diazonium tetrazole treatment. On the other hand, if diazonium tetrazole treatment alters the interactions between the glycine and strychnine sites, the dissociation rate of [³H]strychnine in the presence of excess glycine should be changed. However, disruption by diazonium tetrazole of the interaction of glycine and strychnine sites ought not to alter the dissociation of [³H]strychnine when displaced by nonradioactive strychnine.

To examine these possibilities, we measured the dissociation rate of specific [³H] strychnine binding after treatment with two concentrations of diazonium tetrazole (Fig. 9). The dissociation rate of [³H]strychnine is unaffected by diazonium tetrazole treatment when measured after the addition of unlabeled strychnine at zero time. However, the dissociation rate when displacement is effected with glycine is considerably retarded in the diazonium tetrazole-treated membranes. The half-life for dissociation in control membranes displaced with glycine is 45 sec, while the half-lives using membrane preparations treated with 0.25 mM and 0.75 mM diazonium tetrazole are 56 and 66 sec, respectively.

Effects of guanidine and N-ethylmaleimide on cooperative interactions of glycine and strychnine binding. N-Ethylmaleimide, by alkylating mainly sulfhydryl and amino groupings, alters cooperative interactions of various enzymes (43, 46–48). Guanidine, which disrupts the tertiary and quaternary structure of proteins, conceivably might have similar effects. We examined the influence of N-ethylmaleimide and guanidine on the displacement by glycine of [³H] strychnine binding at concentrations of these reagents which have only small effects on the total amount of specific [³H]strychnine binding (Fig. 10).

Guanidine reduces the Hill coefficient for glycine from 1.7 to 1.26, and N-ethyl-

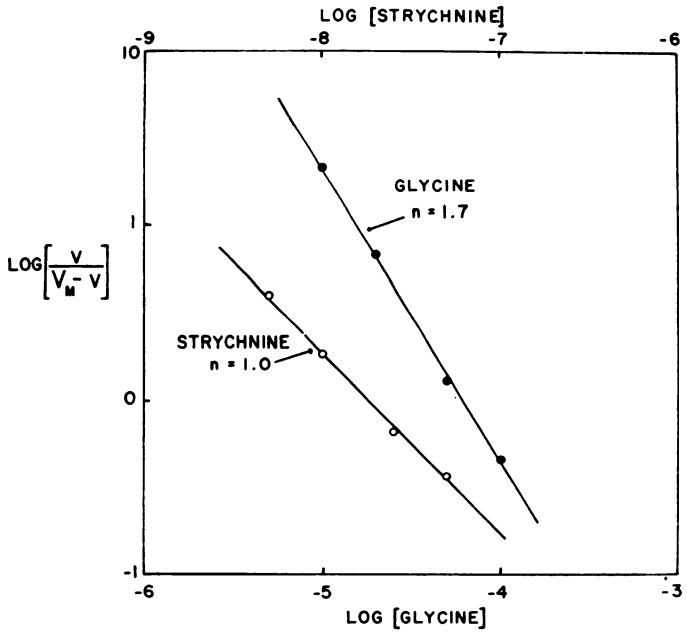

FIG. 6. *Hill plot of displacement of [³H]strychnine binding by nonradioactive glycine and nonradioactive strychnine*

Synaptic membrane suspensions (0.5 mg of protein per tube) were incubated with 2 nM [³H]strychnine (38,000 cpm) and increasing amounts of strychnine or glycine at 4° for 10 min. Nonspecific binding obtained in the presence of 1 mM glycine or 0.1 mM strychnine has been subtracted from all experimental points. Values are the mean of triplicate determinations, which varied less than 15%. The experiment was replicated four times.

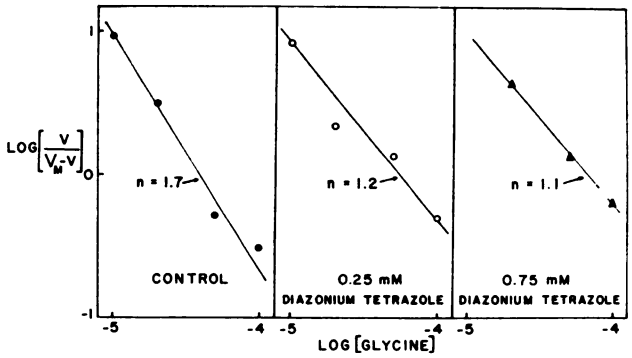

FIG. 7. *Effect of membrane treatment with diazonium tetrazole on displacement of [³H]strychnine by nonradioactive glycine*

Data were plotted according to the Hill equation. Suspensions of synaptic membranes (0.5 mg of protein per milliliter of 0.1 M sodium phosphate buffer, pH 7.1) were allowed to react with diazonium tetrazole (0.25 and 0.75 mM) for 1 hr at 4°. Ten volumes of 0.15 M NaCl were added, and the suspensions were centrifuged for 15 min at 48,000 × *g*. The pellets were resuspended in 2 ml of 0.05 M sodium phosphate buffer, pH 7.1, and 200 mM sodium chloride, and [³H]strychnine binding was then assayed as described in the text with increasing concentrations of glycine. Nonspecific binding obtained in the presence of 1 mM glycine has been subtracted from all experimental values. Values represent the means of triplicate determinations, which varied less than 15%. The experiment was replicated twice. (These concentrations of diazonium tetrazole inhibited [³H]strychnine binding less than 30%.) Hill plots of the displacement of [³H]strychnine by nonradioactive strychnine were unaltered by prior treatment with diazonium tetrazole.

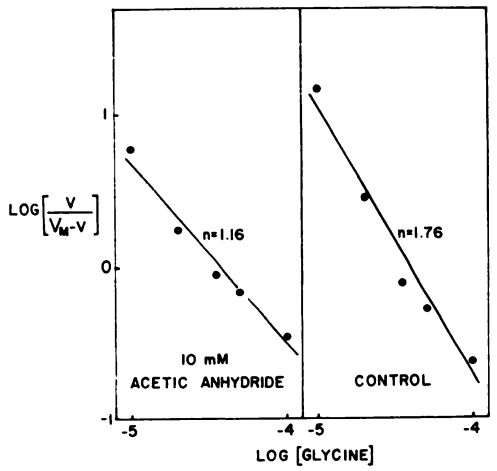

FIG. 8. *Effect of membrane treatment with acetic anhydride on displacement of [³H]strychnine by nonradioactive glycine*

Data were plotted according to the Hill equation. Suspensions of synaptic membrane (0.8 mg of protein per milliliter of 0.1 M sodium phosphate buffer, pH 7.1) were allowed to react with 10 mM acetic anhydride for 1 hr at 4°. Ten volumes of 0.15 M NaCl were added, and the suspensions were centrifuged for 15 min at 48,000 × *g*. The pellets were resuspended in 2 ml of 0.05 M sodium phosphate buffer, pH 7.1, and 200 mM sodium chloride, and [³H]strychnine binding was assayed as described in the text in the presence of increasing concentrations of nonradioactive glycine. Nonspecific binding obtained in the presence of 1 mM glycine has been subtracted from all experimental points. Values represent the means of triplicate determinations, which varied less than 10%.

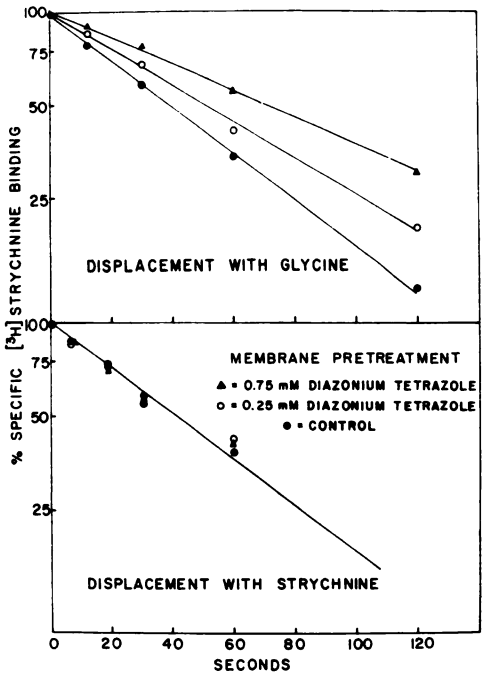

FIG. 9. *Effect of membrane treatment with diazonium tetrazole on dissociation rate of [³H]strychnine-receptor complex*

Membrane suspensions (1.0 mg of protein per assay tube) were treated with 0.25 mM and 0.75 mM diazonium tetrazole as described in Fig. 7, and then the rate of dissociation was determined as described in Fig. 3, using both nonradioactive glycine (upper) and strychnine (lower) as displacers. Values are given as percentages of control binding at zero time for each set of treated membranes. Control binding for the preparations were: no diazonium tetrazole, 0.68 pmole of [³H]strychnine specifically bound per milligram of membrane protein; 0.25 mM diazonium tetrazole, 0.61 pmole/mg; 0.75 mM diazonium tetrazole, 0.48 pmole/mg. Values are the means of triplicate determinations, which varied less than 10%. The experiment was replicated twice.

maleimide lowers it to 0.74. Conceivably these reagents decrease cooperative interactions via their effects on structural links between the glycine and strychnine sites of the receptor.

Effects of pH changes on cooperative interactions between glycine and strychnine binding sites. Changes in pH have been shown previously to alter the sigmoidicity of enzymatic activity and binding curves (43–45, 49). In the strychnine binding system, pH changes affect the cooperativity of glycine displacement (Fig. 11). At pH values below 7.0 the Hill coefficient for glycine displacement is 1.8, while at pH 7.3 and 7.5 the coefficients are 1.20 and 1.05, respectively, indicating a loss in cooperativity with increasing pH. By contrast, when the displacement of [³H]strychnine by nonradioactive strychnine is measured at different pH values, displacement at pH 7.5 appears to increase homotropic cooperative effects, giving a Hill coefficient of 1.2, as compared to the Hill coefficient of 1.0 at pH 6.7.

DISCUSSION

Our previous report presented evidence that specific [³H]strychnine binding to

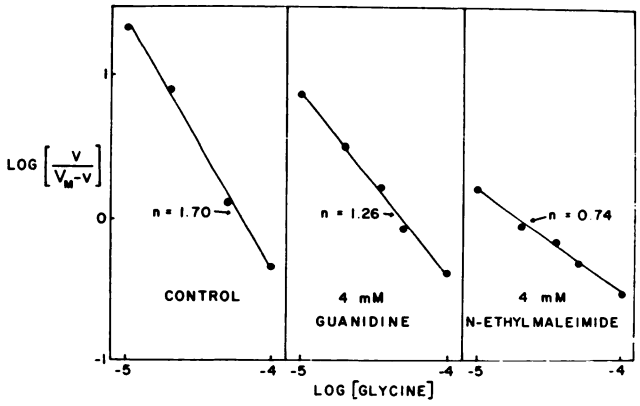

FIG. 10. *Effects of guanidine and N-ethylmaleimide on displacement of [³H]strychnine by nonradioactive glycine*

The data were plotted according to the Hill equation. Synaptic membrane suspensions (0.8 mg of protein per assay tube) were incubated with the indicated concentration of reagent for 30 min at 4° and then assayed for [³H]strychnine binding as described in the text with increasing concentrations of glycine. Nonspecific binding obtained in the presence of 1.0 mM glycine has been subtracted from all experimental points. Values are the means of triplicate determinations, which varied less than 15%. The experiment was replicated twice. Hill plots of the displacement of [³H]strychnine by nonradioactive strychnine were not altered by prior treatment with guanidine, or *N*-ethylamleimide.

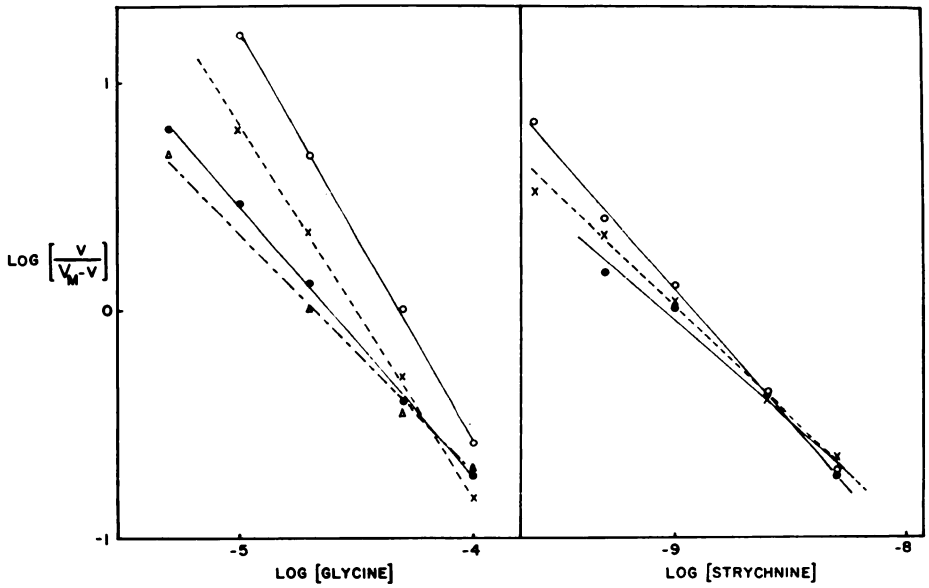

FIG. 11. *Effect of pH on displacement of [³H]strychnine by nonradioactive glycine and strychnine*

Data were plotted according to the Hill equation. Synaptic membrane suspensions (0.4 mg of protein per assay tube) were incubated in 0.05 M sodium phosphate buffer and 200 mM sodium chloride at the indicated pH values with 2 nM [³H]strychnine (38,000 cpm) and increasing amounts of glycine or strychnine at 4° for 10 min. Nonspecific binding obtained in the presence of 1 mM glycine or 0.1 mM strychnine has been subtracted from all experimental points. Values are the means of triplicate determinations, which varied less than 15%. The experiment was replicated three times. Left: ○, pH 6.7, n = 1.82; ×, pH 7.1, n = 1.66; ●, pH 7.3, n = 1.19; △, pH 7.5, n = 1.06. Right: ●, pH 6.6, n = 0.90; ×, pH 7.1, n = 1.00; ○, pH 7.4, n = 1.18.

synaptic membranes of the spinal cord represents an interaction with the postsynaptic receptor for the neurotransmitter effects of glycine (14). The distribution of binding within the central nervous system correlates well with endogenous glycine (16, 17), high-affinity uptake of glycine into unique glycine-accumulating synaptosomes (18–21), and the ability of glycine to mimic natural inhibitory transmission (6–9). Moreover, displacement of specific strychnine binding by several amino acids parallels their glycine-like neurophysiological effects (6), and the highest specific strychnine binding activity occurs in synaptic membrane fractions, consistent with the localization of presumed postsynaptic glycine receptors (14). Because strychnine has negligible affinity for the presynaptic neuronal uptake mechanism for glycine, it is unlikely that this strychnine binding involves presynaptic nerve terminal membranes (19).

In confirmation of previous observations (14), we have found that the association of strychnine with binding sites appears to be bimolecular. These data, along with the presence of a single component in analyses of equilibrium binding at various strychnine concentrations, indicate that binding involves a single population of receptor sites.

The affinity of strychnine for this site is about four orders of magnitude greater than that of glycine. The dissociation constant for glycine (about 25 μM) is similar to values reported for acetylcholine at both nicotinic (for reviews, see refs. 50 and 51) and muscarinic (52–54) receptors. It is thus conceivable that affinity of approximately this magnitude may be a general property for neurotransmitter at their receptor sites. Association kinetics is bimolecular, with a rate constant of 1.0×10^7 M^{-1} sec^{-1}, while dissociation of the strychnine-receptor complex is first-order, with a rate constant of 1.54×10^{-2} sec^{-1}. The dissociation constant (k_2/k_1) of 1.54×10^{-9} M is similar to that obtained from equilibrium data.

In the present study we have confirmed our earlier finding that the affinity for binding is relatively specific and that amino acids with glycine-like neurophysiological activity are the most efficient ones in competing for receptor binding. Interestingly, phenylalanine and tryptophan, which have some affinity for the receptor, are thought to be biosynthetic precursors of strychnine (55).

Monovalent cations increase specific and decrease nonspecific strychnine binding. This results in considerably higher specific to nonspecific binding ratios, which greatly increase the sensitivity and ease of binding assays. The increased binding with high salt concentrations might be due to an unmasking of "buried" receptor sites, as has been observed for the insulin receptor (56). The decrease in nonspecific binding elicited by these cations may indicate that a significant portion of the nonspecific binding represents ionic interactions which are displaced by monovalent cations.

The inhibition of [^{3}H]strychnine binding by urea and guanidine occurs at lower concentrations of these reagents than those that normally interfere with the tertiary structure of proteins, and may indicate selective effects on the binding interactions. It is possible, however, that these reagents disrupt the quaternary structure of the membrane at such low concentrations. Urea has been shown to reverse the inhibition of aspartate transcarbamylase by CTP at 0.8 M by disrupting the quaternary structure of the enzyme (43, 44, 49). However, these reagents may inhibit strychnine binding either directly or by a combination of effects.

The detergents Triton X-100 and deoxycholate decrease binding in concentrations which solubilize membrane proteins. This suggests that the fall in binding results from solubilization of strychnine binding components of the membrane. This conclusion is supported by our demonstration of specific [^{3}H]strychnine binding to reprecipitated extracts obtained after treating synaptic membrane fractions of rat spinal cord with 0.4% deoxycholate or 1% Triton X-100, centrifugation for 2 hr at 100,000 $\times$ g, and reprecipitating solubilized material after removal of the detergent.[3] In such preparations we recover about 10% of the

[3] A. B. Young and S. H. Snyder, manuscript in preparation.

strychnine-binding capacity of the intact synaptic membranes. This low recovery may be due to incomplete precipitation of the soluble material or to direct influences of the detergents on strychnine binding.

Some reagents differentially alter the ability of glycine or nonradioactive strychnine to compete with [^{3}H]strychnine binding. Dinitrofluorobenzene and tetranitromethane appear simply to inhibit total strychnine binding without influencing the proportional displacement of the remaining binding by glycine or nonradioactive strychnine. In contrast, diazonium tetrazole and acetic anhydride inhibit total strychnine binding only to a limited extent but decrease the ability of glycine and nonradioactive strychnine to displace [^{3}H]strychnine. Furthermore, the effects on displacement are different for glycine and strychnine. After treatment with 2.5 mM diazonium tetrazole, glycine no longer displaces [^{3}H]strychnine, but nonradioactive strychnine still displaces 50% of the binding. With acetic anhydride treatment (25 mM) glycine displaces strychnine binding only 30%, whereas nonradioactive strychnine can still displace 84% of the binding.

Protection experiments also support the differentiation of glycine and strychnine binding sites. If diazonium tetrazole acts at a binding site common to strychnine and glycine, both might be expected to protect against inhibition by the reagent. If the binding sites are separate for strychnine and glycine, only one might protect. Since only glycine protects against diazonium tetrazole treatment, strychnine and glycine apparently bind to distinct sites.

The differential effects of diazonium tetrazole and acetic anhydride suggested that strychnine and glycine may interact in an allosteric fashion (29–32). Accordingly, experiments were analyzed using the Hill equation (42). After treatment of synaptic membranes with either diazonium tetrazole or acetic anhydride, the Hill coefficients of displacement curves are reduced to 1.2 from values of 1.7 for controls. Prior treatment with diazonium tetrazole retards the dissociation of the strychnine-receptor complex when glycine is used as a displacer, but not when nonradioactive strychnine is used. Thus these reagents seem to inhibit the cooperative interactions of the glycine and strychnine binding sites.

N-Ethylmaleimide, which modifies mainly sulfhydryl and amino groups, does not inhibit either total or specific [^{3}H]strychnine binding significantly. However, it does affect the cooperativity between glycine and strychnine binding sites, causing the Hill coefficient of glycine displacement curves to fall from 1.7 to 0.74.

The results with various protein-modifying reagents suggest that histidyl, sulfhydryl, and amino groups are involved in the cooperative interactions of strychnine and glycine sites but may not be essential for the binding process directly. This is evidenced by the differential effects of diazonium tetrazole and acetic anhydride on glycine displacement of strychnine binding and by the effects of both these reagents and *N*-ethylmaleimide on the slopes of the Hill plots of glycine displacement curves. In contrast, tyrosyl and perhaps tryptophanyl groups may be involved directly with strychnine binding, as indicated by the direct effects of tetranitromethane and dinitrofluorobenzene on total strychnine binding. However, the nonspecificity of these reagents and the crude nature of the membrane preparation preclude any conclusive interpretations.

These studies with protein-modifying reagents were performed at pH 7.1. At other pH values the reagents may have different effects, since their reactions with various amino acid residues in proteins are known to be pH-dependent. The inhibitory effects of the reagents on specific strychnine binding are almost certainly not secondary to a direct reaction of the reagent with glycine or strychnine, because these experiments have been replicated after extensive washing of the treated membranes before assaying for strychnine binding. Furthermore, most of the reagents that did affect binding did so only at high concentrations. After 20-fold dilution they had virtually no effect on specific binding, again indicating that the initial wash after preliminary treatment is sufficient to eliminate a direct reaction with the ligand as a cause of the inhibitory effect.

The studies with protein-modifying reagents first suggested the possibility of glycine and strychnine binding at two separate but mutually interacting sites. Additional studies of glycine and strychnine competition with [^{3}H]strychnine binding indicated that strychnine competes directly with radiolabeled strychnine but that glycine competition possesses aberrant kinetics. Double-reciprocal analysis of strychnine binding in the presence of glycine revealed a deviation from linearity, with curves becoming concave upward. Furthermore, Hill plots of glycine displacement of bound strychnine gave Hill coefficients of 1.7, indicating a heterotropic cooperative interaction. This cooperativity could be altered by protein-modifying reagents, pH changes, and denaturants such as guanidine HCl. It is interesting that such treatments alter the cooperativity of the interaction of glycine with strychnine binding without altering the absolute magnitude of the maximal inhibitory effect, i.e., without desensitization of strychnine binding to the inhibitory effects of glycine. Similar effects have been found with the classic allosteric enzyme systems aspartate transcarbamylase (43) and threonine dehydratase (46) and may be due to a weakening of the interaction between the different sites, which would change the cooperativity and perhaps the sensitivity of the inhibitory effect without altering the maximal effect.

The nature of the apparently distinct glycine and strychnine binding sites is unclear. Neurophysiological studies have demonstrated cooperative interactions between γ-aminobutyric acid and its antagonist picrotoxin (26, 27, 57) and have indicated that more than one transmitter molecule may be necessary to activate one receptor in the case of γ-aminobutyric acid (24–27), acetylcholine (28, 58, 59), and glycine (24, 25). Cooperativity has also been demonstrated for nicotinic cholinergic receptor binding (60). Neurotransmitters may interact with recognition sites on the postsynaptic receptor, triggering a change in ionic conductance at an intimately related ionophore site. Inhibitory transmitters such as glycine are thought to affect selectively the permeabilty of the postsynaptic membrane to potassium and chloride (61, 62). Inhibitory conductance changes are elicited to varying extents by intracellular manipulations of the chloride, bromide, and iodide concentrations. Our studies of the differential effects of anions on [^{3}H]strychnine binding (63) are consistent with the hypothesis that strychnine interacts with the glycine-related ionophore site. Thus anions which are capable of functioning like chloride neurophysiologically (61, 62) also inhibit [^{3}H]strychnine binding. Similarly, anions which seem too large (in terms of hydration radius) can neither substitute for chloride neurophysiologically nor inhibit strychnine binding.

The technique described here for measuring strychnine interactions with membrane components provides a direct biochemical method for studying interactions of neurotransmitter agonists and antagonists. Conceivably, for transmitters other than glycine, antagonists and agonists may also interact cooperatively at the postsynaptic receptor.

REFERENCES

1. Aprison, M. H. & Werman, R. (1965) *Life Sci.*, **4**, 2075–2083.
2. Graham, L. T., Jr., Shank, R. P., Werman, R. & Aprison, M. H. (1967) *J. Neurochem.*, **14**, 465–472.
3. Davidoff, R. A., Graham, L. T., Jr., Shank, R. P., Werman, R. & Aprison, M. H. (1967) *J. Neurochem.*, **14**, 1025–1031.
4. Matus, A. I., & Dennison, M. E. (1971) *Brain Res.*, **32**, 195–197.
5. Hökfelt, T. & Ljungdahl, A. (1971) *Brain Res.*, **32**, 189–194.
6. Curtis, D. R., Hösli, L. & Johnston, G. A. R. (1968) *Exp. Brain Res.*, **6**, 1–18.
7. Kelly, J. S. & Krnjevic, K. (1969) *Exp. Brain Res.*, **9**, 155–163.
8. Galindo, A., Krnjevic, K. & Schwartz, S. (1967) *J. Physiol. (London)*, **192**, 359–377.
9. Werman, R., Davidoff, R. A. & Aprison, M. H. (1968) *J. Neurophysiol*, **31**, 81–95.
10. Dusser de Barenne, J. G. (1933) *Physiol. Rev.*, **13**, 325–335.
11. Bradley, K., Easton, D. M. & Eccles, J. C. (1953) *J. Physiol. (London)*, **122**, 474–488.
12. Owen, A. G. W. & Sherrington, C. S. (1911) *J. Physiol. (Lond.)*, **43**, 232–241.

13. Curtis, D. R., Duggan, A. W. & Johnston, G. A. R. (1971) *Exp. Brain Res.*, **12**, 547–565.
14. Young, A. B. & Snyder, S. H. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 2832–2836.
15. Snyder, S. H., Young, A. B., Bennett, J. P. & Mulder, A. H. (1973) *Fed. Proc.*, **32**, 2039–2047.
16. Aprison, M. H., Shank, R. P., Davidoff, R. A. & Werman, R. (1968) *Life Sci.*, **7**, 583–590.
17. Aprison, M. H., Shank, R. P. & Davidoff, R. A. (1969) *Comp. Biochem. Physiol.*, **28**, 1345–1355.
18. Logan, W. J. & Snyder, S. H. (1971) *Nat. New Biol.*, **234**, 297–299.
19. Johnston, G. A. R. & Iversen, L. L. (1971) *J. Neurochem.*, **18**, 1951–1961.
20. Logan, W. J. & Snyder, S. H. (1972) *Brain Res.*, **42**, 413–431.
21. Arregui, A., Logan, W. J., Bennett, J. P. & Snyder, S. H. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 3485–3489.
22. Krnjevic, K., Randic, M. & Straughan, D. W. (1966) *J. Physiol. (Lond.)*, **184**, 78–105.
23. Biscoe, T. J. & Curtis, D. R. (1967) *Nature*, **214**, 914–915.
24. Werman, R. (1969) *Comp. Biochem. Physiol.*, **30**, 997–1017.
25. Werman, R. (1972) *Assoc. Res. Nerv. Ment. Dis.*, **50**, 147–180.
26. Brookes, N. & Werman, R. (1973) *Mol. Pharmacol.*, **9**, 571–579.
27. Brookes, N. & Werman, R. (1970) *Isr. J. Med. Sci.*, **6**, 322.
28. Karlin, A. (1967) *J. Theor. Biol.*, **16**, 306–320.
29. Monod, J., Changeux, J.-P. & Jacob, F. (1963) *J. Mol. Biol.*, **6**, 306–329.
30. Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.*, **12**, 88–118.
31. Koshland, D. E., Jr., Némethy, G. & Filmer, D. (1966) *Biochemistry*, **5**, 365–385.
32. Wyman, J. (1964) *Adv. Protein Chem.*, **19**, 223–286.
33. Hoare, D. G. & Koshland, D. E., Jr. (1967) *J. Biol. Chem.*, **242**, 2447–2453.
34. Sokolovsky, M., Riordan, J. R. & Vallee, B. L. (1966) *Biochemistry*, **5**, 3582–3589.
35. Cuatrecasas, P., Fuchs, S. & Anfinsen, C. B. (1968) *J. Biol. Chem.*, **243**, 4787–4798.
36. Horton, H. R. & Koshland, D. E., Jr. (1965) *J. Am. Chem. Soc.*, **87**, 1126–1132.
37. Barman, T. E. & Koshland, D. E., Jr. (1967) *J. Biol. Chem.*, **242**, 5771–5776.
38. Horton, H. R., Kelly, H. & Koshland, D. E., Jr. (1965) *J. Biol. Chem.*, **240**, 722–724.
39. Sokolovsky, M. & Vallee, B. L. (1966) *Biochemistry*, **5**, 3574–3581.
40. Cuatrecasas, P. (1970) *J. Biol. Chem.*, **245**, 574–584.
41. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
42. Robinson, J. D. (1967) *Biochemistry*, **6**, 3250–3258.
43. Weitzman, P. D. & Wilson, I. B. (1966) *J. Biol. Chem.*, **241**, 5481–5488.
44. Gerhart, J. C. & Pardee, A. B. (1964) *Fed. Proc.*, **23**, 727–735.
45. Changeux, J.-P. (1962) *J. Mol. Biol.*, **4**, 220–225.
46. Changeux, J.-P. (1961) *Cold Spring Harbor Symp. Quant. Biol.*, **26**, 313–318.
47. Gerhart, J. C. & Schachman, H. K. (1965) *Biochemistry*, **4**, 1054–1062.
48. Tobin, T., Banerjee, S. P. & Sen, A. K. (1970) *Nature*, **225**, 745–746.
49. Gerhart, J. C. & Pardee, A. B. (1962) *J. Biol. Chem.*, **237**, 891–896.
50. Porter, A. & O'Connor, M. (1970) *Ciba Foundation Symposium on Molecular Properties of Drug Receptors*, Churchill, London.
51. Potter, L. T. & Molinoff, P. B. (1972) in *Perspectives in Neuropharmacology* (Snyder, S. H., ed.), p. 9, Oxford University Press, New York.
52. Burgen, A. S. V., Hiley, C. R. & Young, J. M. (1974) *Br. J. Pharmacol.*, in press.
53. Yamamura, H. I. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 1725–1729.
54. Yamamura, H. I., Kuhar, M. J., Greenberg, D. & Snyder, S. H. (1974) *Brain Res.*, **66**, 541–546.
55. Woodward, R. B. (1955) *Experientia (Suppl.)*, **2**, 213–228.
56. Cuatrecasas, P. (1971) *J. Biol. Chem.*, **246**, 7265–7274.
57. Takeuchi, A. & Takeuchi, N. (1969) *J. Physiol. (Lond.)*, **205**, 377–391.
58. Changeux, J.-P. & Podleski, T. R. (1968) *Proc. Natl. Acad. Sci. U. S. A.*, **59**, 944–950.
59. Changeux, J.-P., Meunier, J.-C., Olsen, R. W., Weber, M., Bourgeois, J. P., Popot, J.-L., Cohen, J. B., Hazelbauer, G. L. & Lester, H. A. (1973) in *Drug Receptors* (Rang, H. R., ed.), p. 273, University Park Press, Baltimore.
60. Eldefrawi, M. E. & Eldefrawi, A. T. (1973) *Biochem. Pharmacol.*, **22**, 3145–3150.
61. Eccles, J. C. (1964) *The Physiology of Synapses*, Academic Press, New York.
62. Eccles, J. C. (1966) *Ann. N. Y. Acad. Sci.*, **137**, 473–495.
63. Young, A. B. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, in press.