unfavorable steric interactions.

With the octapeptide fragment of the natural substrate at the active site of human renin as a template, models for several inhibitors discussed here were constructed at the active site of human renin. Inhibitors representative of different structural classes were chosen for construction. These inhibitors are RIP, **9**, **18**, and **28**. The peptide backbone and the amino acid side chains of the natural substrate or of pepstatin were used directly where they were identical with the inhibitor. For the phenylalanines in the P1 and P1′ positions of RIP, the χ_1 and χ_2 angles for the corresponding residues of the model for the natural substrate were used to orient the phenylalanines in the preliminary model. The phenylalanine side chains were then adjusted by rotating the side chain around the Cα–Cβ bond (χ_1) and the Cβ–Cγ bond (χ_2) to minimize unfavorable steric contacts.

After preliminary models were constructed of pepstatin, the natural substrate and the inhibitors, the structures were refined by using the energy refinement program CHARMM.[44] The refinement was done in a manner similar to that used for the structure of human renin.[18] Constraints were placed on the movement of atoms from the initial positions and reduced every 25 cycles for the first 100 cycles. Another 100 cycles were completed without constraints on either the protein or the substrate.

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Limited Nerve Impulse Blockade by "Leashed" Local Anesthetics

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To measure the depth of the local anesthetic binding site within the neuronal membrane, biotin-containing polyethylene glycols having zero, three, and six ethylene glycol subunits were added to the *p*-amino termini of tetracaine and procaine, thereby interposing a pharmacologically inert "spacer" molecule between the local anesthetic and the biotin moiety. These biotinyl–local anesthetic derivatives produced "tonic" inhibition of the compound action potential of split, desheathed frog sciatic nerves in a concentration-dependent, reversible manner. However, no inhibition of the action potential occurred when sufficient avidin, a 66 000-MW protein that binds four biotins, was present to bind and anchor the biotin-containing end of each derivative outside the plasma membrane. Increasing the "leashed" anesthetic derivative's concentration to 4 times that which reduced impulse height by 50% in the absence of avidin still produced no detectable block when equimolar avidin was present. Apparently, the "spacer" in the derivative compound was too short to permit the avidin-complexed anesthetic to reach its site of action on the sodium channel. In a similar fashion, the local anesthetic derivatives produced "use-dependent" block when drug-treated nerves were stimulated at 40 Hz in the absence of equimolar avidin, but failed to produce "use-dependent" block when equimolar avidin was present. In common with others, we assume that tertiary amine local anesthetics may reach their binding site via hydrophobic (transmembrane) pathways without necessarily entering the cytoplasm. Thus, since our longest local anesthetic derivative, that containing six ethylene glycol subunits, placed the local anesthetic group a maximum of 15–18 Å from the surface of the avidin moiety, we conclude that the local anesthetic binding site for block of sodium channels of amphibian nerve must be ≳15 Å from the outer surface of the plasma membrane.

Since local anesthetics were first identified as having an effect on nerves, several notable advances have been made toward defining their locus of action. The first advance was the demonstration by Taylor in 1959 that, under voltage clamp conditions, the primary effect of procaine on the squid axon was to decrease the sodium current activated by depolarizations.[1]

Experiments with permanently charged, quaternary amine derivatives of local anesthetics and with tertiary amine local anesthetics at different axoplasmic and extracellular pH values demonstrated a general lack of potency of charged local anesthetics applied to the external surface of neuronal membranes.[2,3] This contrasted markedly with both the potency of permanently uncharged local anesthetics (e.g., benzocaine) and of tertiary amine local anesthetics (at physiological pH) when applied on either side of the plasma membrane, and the potency of charged local anesthetics when directly applied inside the

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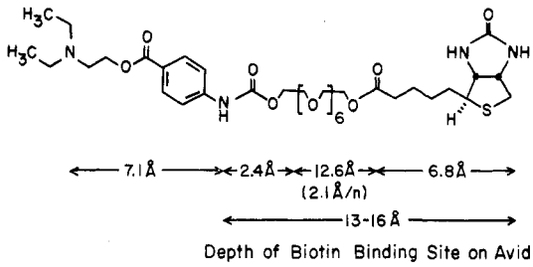
Scheme I. Molecular Dimensions and Structures of Local Anesthetic Derivatives[a]



[a] The dimensions shown, for P-6-Bio, are the linear distances along the long axis of a three-dimensional model of this molecule.[19] Depth of local anesthetic binding site on avidin calculated with data from Green et al.[10]

neurons.[4] These studies demonstrated the critical role played by membrane permeation in determining the potency of protonatable local anesthetics, but did not reveal their loci of action on the sodium channel itself.

There is evidence for a local anesthetic binding site within the aqueous pore of the sodium channel. Due to the limited lipid solubility of sodium ions, the observed antagonism by sodium ions of the local anesthetic-induced inhibition of sodium currents likely occurs within an aqueous region of the channel,[5] although this interaction may also be allosteric and occur by binding at separate sites. On the other hand, a variety of other, hydrophobic drugs modify sodium channel function and inhibit nerve impulses. These include such uncharged and highly lipid-soluble inhibitory agents as halothane, benzocaine, and carbon tetrachloride[4,6,7] and the lipid-soluble "activator" toxins, like veratridine and batrachotoxin, which both open sodium channels and antagonize their block by local anesthetics. Whether these compounds act at the same binding site(s) as the more typical tertiary amine local anesthetics and their quaternary derivatives is not known.

Mindful of these concerns, we sought a way in which to limit the permeation of a local anesthetic through membranes without modifying the protonation equilibria of the tertiary amine moiety to measure the depth of the local anesthetic binding site within the neuronal membrane. We chose chemical binding of D-biotin to the aromatic amine of procaine or tetracaine via a "spacer" molecule of defined and variable length. Avidin, a protein normally found in egg whites, binds biotin with high affinity ($K_a = 10^{15}$ M^{-1}). It is a large (66 000 MW), basic, globular protein and is most probably quite membrane-impermeant.[8] Therefore, we anticipated that avidin would limit the membrane penetration of biotin-conjugated, local anesthetic derivatives. By varying the length of the "spacer", we sought to define and control the "leashed" anesthetic's depth of membrane penetration (Scheme I) and, correspondingly, its potency in blocking sodium channels. We chose ethylene glycol polymers as our spacers, since ether linkages are well known to increase the membrane permeability of nonelectrolyte polymers while maintaining their aqueous solubility.[9] We predicted that such modifications would limit anesthetic access to binding sites reached via hydrophobic (transmembrane) pathways, a likely route by which local anesthetics reach their binding site when applied to nerve trunks in vitro or in vivo. We thought it unlikely that tertiary amine anesthetics *must* pass first through cytoplasm to reach their binding site. If such were the case, "spacers" would be required of such great length that our measurements would likely be impracticable. Our results further narrow the possible sites within the sodium channel where the anesthetic binds. Similar techniques exploiting avidin's affinity for biotin have been used in the past to define the depth of the biotin-binding site on avidin,[10] to purify the adrenocorticotropic hormone (ACTH) and insulin receptors,[11,12] and to characterize the binding of curarimimetic toxin from cobra venom to the nicotinic acetylcholine receptor.[13]

Methods

Chemical Syntheses and Analyses. Syntheses of the derivatives of the local anesthetics followed conceptually straightforward routes. These syntheses are, however, technically difficult due to the difficulty in purifying products and intermediates and to the lability of ester links under certain circumstances. To facilitate the discussion, we introduce an abbreviated nomenclature. The molecules are designated by a two-letter, one-number code. The first letter describes the local anesthetic, the letters P and T referring to procaine and tetracaine, respectively. The number, *n*, refers to the number of ethylene glycol ($-OCH_2CH_2-$) units in the chain. The second set of letters refers to the atom or chemical moiety attached to the end of the chain: H for hydrogen, Bn for benzyl, or Bio for biotin (See Scheme I).

The molecules with the shortest possible chain in this series are the tetracaine and procaine amides of biotin, T-0-Bio and P-0-Bio, respectively. As shown in Scheme II, eq 1, T-0-Bio (compound **1**) was prepared in 68% yield by the reaction of tetracaine (compound **3**) with biotinyl chloride (compound **4**) and triethylamine in dimethylformamide. P-0-Bio (eq 2, compound **2**) was prepared in 61% yield by treating procaine (compound **5**) with an excess of D-biotin (compound **6**) and dicyclohexylcarbodiimide (DCC) in the presence of a catalytic amount of (*N,N*-dimethylamino)pyridine (DMAP) in methylene chloride.

The synthesis of the longer chain derivatives P-3-Bio (compound **10a**) and P-6-Bio (compound **10b**) is illustrated in eq 3 of Scheme II. The preparation of the triethylene glycol monobenzyl ether has been described by Reimschneider and Schneider.[14] The monobenzyl ether of hexaethylene glycol, Bn-6-H (compound **7b**), was prepared in 30% yield by treating hexaethylene glycol with potassium *tert*-butoxide and benzyl chloride. Each monobenzyl ether was treated with *N,N*-dimethylaniline and trichloromethyl chloroformate in benzene to give their chloroformates, which were not isolated but reacted directly with procaine in pyridine solution. The carbamates P-3-Bn (compound **8a**) and P-6-Bn (compound **8b**) were prepared in 98% and 100% yields, respectively, by this procedure.

Hydrogenolysis of P-3-Bn and P-6-Bn with 10% Pd/C in ethanol provided the alcohols P-3-H (compound **9a**) and P-6-H (compound **9b**) in 88% and 89% yields, respectively. Each of these compounds was esterified with D-biotin, by using DCC and DMAP in methylene chloride, to give P-3-Bio and P-6-Bio in 85% and 89% yields, respectively.

General Considerations. Ultraviolet (UV) measurements were made with a Perkin-Elmer Model 552 spectrophotometer. Infrared (IR) measurements were made with a Perkin-Elmer Model 598 spectrophotometer and calibrated with a polystyrene film. ^1H and ^{13}C spectra were measured by nuclear magnetic

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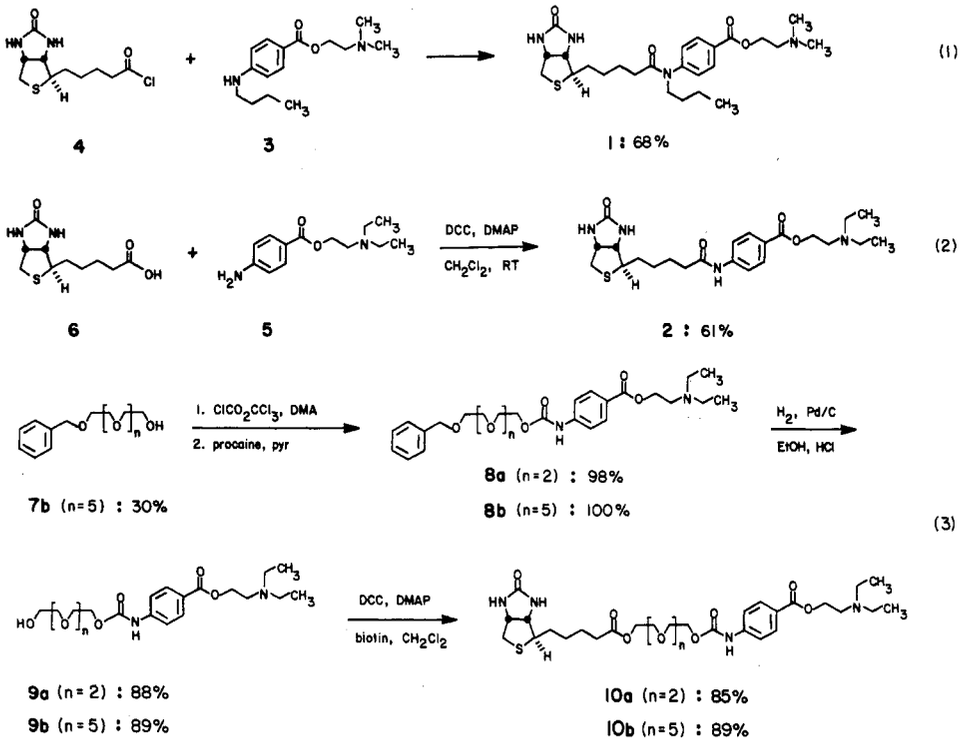
Scheme II. Synthesis of Biotinylated Local Anesthetics from Tetracaine and Procaine



resonance (NMR) on a Bruker Model WM 300 spectrometer at 300.1 and 75.5 MHz, respectively. Chemical shifts were reported relative to a tetramethylsilane internal standard. Mass spectra (fast atom bombardment) were measured on a Vacuum Generator Model VG 7070E spectrometer. Elemental analyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, MI. HPLC analyses were carried out on a Waters Associates system equipped with a differential ultraviolet detector operating at 254 nm, using a Waters Radial-PAK C-18 column (5 mm × 10 cm, 10-μm particle size). The mobile phase was NaOAc/HOAc/H_2O (4.0 g:40 mL:1000 mL) containing 60–90% acetonitrile. Thin-layer chromatography (TLC) was carried out on Merck silica gel 60-F254 glass-backed plates (0.25-mm layer thickness). Column chromatography was performed on Merck silica gel 60 (230-400 mesh). A Branson Model D-100 sonicator was used where specified. Melting points (mp) were determined on a Thomas-Hoover capillary melting point apparatus and are reported uncorrected. All reactions were carried out in oven-dried glassware under an argon atmosphere. Methylene chloride, pyridine, benzene, and hexaethylene glycol were dried over 4-Å molecular sieves before use. All other chemicals were commercially available, reagent grade, and were used without purification.

[3a*S*-(3aα,4β,6aα)]-2-(Diethylamino)ethyl 4-[[5-(Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]amino]benzoate (P-0-Bio) (Scheme II, Compound 2). Biotin (0.488 g, 2.0 mmol), procaine (0.236 g, 1.0 mmol), 4-(*N*,*N*-dimethylamino)pyridine (0.04 g, 0.33 mmol), dicyclohexylcarbodiimide (0.921 g, 4.46 mmol), and methylene chloride (20 mL) were placed in a 25-mL round-bottomed flask equipped with an inlet for argon and magnetic stirring bar. The suspension was stirred at room temperature for 48 h and then poured into ethyl acetate (100 mL) containing water (2 mL). After stirring for 10 min, the suspension was filtered and the filtrate concentrated in vacuo. The residue was chromatographed (silica gel 60, EtOAc/MeOH (3:2)) and gave, after isolation, 0.28 g (61% yield) of P-0-Bio as a white solid. For analysis and biological testing, a sample was crystallized from aqueous acetone: mp 206 °C scinters, 211–213 °C dec; UV max (95% EtOH) 273 (ϵ 21 700), 205 nm (15 800); IR ($CHCl_3$) 3478 (N—H), 2940 (C—H), 1705 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.59 (br s, 1), 7.98 (d, 2, J = 8.7 Hz), 7.69 (d, 2, J = 8.7 Hz), 6.50 (br s, 1), 5.34 (br s, 1), 4.52 (m, 1), 4.37 (t, 2, J = 6.2 Hz), 4.33 (m, 1), 3.15 (q, 1, J = 4.5 Hz), 2.91 (m, 1), 2.86 (m, 1), 2.70 (m, 1), 2.67 (q, 4, J = 7.1 Hz), (dd, 1, J = 7.3 Hz), 1.26–1.08 (m, 6), 1.07 (t, 6, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 172.30, 166.28, 164.31, 142.89, 130.73, 125.29, 118.92, 63.38, 61.78, 60.29, 55.78, 51.15, 47.85, 40.60, 36.94, 28.20, 28.02, 25.64, 12.10; MS, m/z 462 M^+, 346, 306. Anal. Calcd for $C_{23}H_{34}N_4O_4S$: C, 59.72; H, 7.41; N, 12.11; S, 6.93. Found: C, 59.80; H, 7.62; N, 12.09; S, 6.88.

2-(Diethylamino)ethyl 4-[(1-Oxo-12-phenyl-2,5,8,11-tetraoxadodec-1-yl)amino]benzoate (P-3-Bn) (Scheme II, Compound 8a). Trichloromethyl chloroformate (1.74 g, 9.36 mmol) and benzene (15 mL) were placed in a 100-mL round-bottomed flask equipped with an addition funnel, magnetic stirring bar, and inlet for argon. The solution was cooled in an ice bath, and a solution of triethylene glycol monobenzyl ether (14) (4.09 g, 17.0 mmol) and *N*,*N*-dimethylaniline (2.27 g, 18.7 mmol) in benzene (30 mL) was added dropwise over a period of 15 min. The resulting white suspension was stirred at 4 °C for 3 h. The benzene was removed quickly in vacuo, and the residue was cooled in an ice bath. A solution of procaine (4.02 g, 17.0 mmol) in pyridine (40 mL) was added in one portion. The resulting yellow suspension was stirred at 0 °C for 10 min and then at room temperature for 6 h. The orange solution was stirred with saturated $NaHCO_3$ solution (200 mL) and extracted with ethyl acetate (3 × 200 mL). The combined organic extracts were washed with saturated NaCl solution (1 × 100 mL), dried ($MgSO_4$), and concentrated in vacuo to give 9.81 g of a yellow oil, which was extracted with *n*-pentane (to remove dimethylaniline) and then heated to 85 °C at 0.1 torr. The resulting yellow oil (8.4 g, ca. 98% yield) was nearly pure by TLC (silica gel, EtOAc/MeOH (1:1), R_f 0.55). This material was used in the next reaction without further purification. For analysis and biological testing, a sample was chromatographed (silica gel 60, EtOAc/MeOH (4:1)) to give P-3-Bn as a colorless oil: UV max (95% EtOH) 270 nm (ϵ 5950); IR ($CHCl_3$) 3440 (N—H), 2950 (C—H), 1745 (urethane C=O), 1717 (ester C=O), 1615 (C=C), 1530 (C—N), 1418 (C—O), 1280 (C—O), 1180 (C—O), 1120 (C—O), 1075 (C—O—C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.97 (d, 2, J = 8.7 Hz), 7.42 (d, 2, J = 8.7 Hz), 7.34–7.27 (m, 5), 7.08 (br s, 1), 4.57 (s, 2), 4.56–4.32 (m, 4), 3.77–3.64 (m, 10), 2.85 (t, 2, J = 6.3 Hz), 2.63 (q, 4, J = 7.1 Hz), 1.07 (t, 6, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 166.18, 153.11, 142.50, 131.60, 130.86, 124.78, 117.54, 113.75, 72.37, 70.49, 70.17, 69.20, 64.12, 63.24, 61.63, 51.08, 47.81, 12.07; MS, m/z 502 (M^+). Anal. Calcd for $C_{27}H_{38}N_2O_7$: C, 64.52; H, 7.72; N, 5.57. Found: C, 64.30; H, 7.55; N, 5.34.

2-(Diethylamino)ethyl 4-[[[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]carbonyl]amino]benzoate (P-3-H) (Scheme II, Compound 9a). Unpurified P-3-Bn (6.24 g, ca. 12.4 mmol), 10% Pd on activated carbon (1.10 g), absolute ethanol (50 mL), water

(10 mL), and 12 N HCl (2 mL) were placed in a Parr apparatus and hydrogenated at 50 psi for 48 h. The reaction mixture was filtered and concentrated in vacuo. The residue was partitioned between ethyl acetate (100 mL) and saturated $NaHCO_3$ solution (50 mL). The aqueous layer was extracted with ethyl acetate (3 × 50 mL), and the combined organic extracts were washed with saturated NaCl solution (1 × 50 mL). The solution was dried ($MgSO_4$) and concentrated in vacuo to give P-3-H as a colorless oil (4.5 g, 88% yield). For analysis and biological testing, a sample was chromatographed (silica gel 60, EtOAc/MeOH (3:2)): UV max (95% EtOH) 270 nm (ϵ 6000); IR ($CHCl_3$) 3455 (OH), 2990 (CH), 1750 (urethane C=O), 1720 (ester C=O), 1620 (C=C), 1535 (CN), 1420 (C—O), 1282 (C—O), 1120 (C—O), 1080 (C—O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.97 (d, 2, J = 8.7 Hz), 7.80 (br s, 1), 7.47 (d, 2, J = 8.7 Hz), 4.39–4.31 (m, 4), 3.77–3.63 (m, 10), 2.85 (t, 2, J = 6.2 Hz), 2.63 (q, 4, J = 7.1 Hz), 1.07 (t, 6, 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 166.18, 153.11, 142.50, 131.60, 130.86, 124.78, 117.54, 113.75, 72.37, 70.49, 70.17, 69.20, 64.12, 63.24, 61.63, 51.08, 47.81, 12.07; MS, m/z 412 (M^+). Anal. Calcd for $C_{20}H_{32}N_2O_7$: C, 58.24; H, 7.82; N, 6.29. Found: C, 58.00; H, 7.92; N, 6.67.

[3a*S*-(3aα,4β,6aα)]-2-[2-[2-[[[[4-[[2-(Diethylamino)ethoxy]carbonyl]phenyl]amino]carbonyl]oxy]ethoxy]ethoxy]ethyl Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanoate (P-3-Bio) (Scheme II, Compound 10a). Biotin (1.35 g, 5.53 mmol), P-3-H (1.16 g, 2.81 mmol), dicyclohexylcarbodiimide (0.72 g, 3.49 mmol), 4-(*N*,*N*-dimethylamino)pyridine (0.06 g, 0.49 mmol), and methylene chloride (13 mL) were sealed under an argon atmosphere inside a 25-mL round-bottomed flask. The reaction mixture was sonicated at 40 °C for 68 h and then poured into ethyl acetate (50 mL) and filtered. The filtrate was concentrated in vacuo and the residue chromatographed (silica gel 60, EtOAc/MeOH (1:1)) to give P-3-Bio (1.53 g, 85% yield) as a colorless oil: UV max 270 (ϵ 29 000), 205 nm (27 800); IR ($CHCl_3$) 3480 (N—H), 3440 (N—H), 2975 (C—H), 1720 (C=O), 1610 (C=C), 1530 (C—N), 1280 (C—O), 1120 (C—O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.95 (d, 2, J = 8.7 Hz), 7.52 (d, 2, J = 8.7 Hz), 6.40 (br s, 1), 5.40 (br s, 1), 4.52 (m, 1), 4.36 (t, 2, J = 6.2 Hz), 4.38–4.13 (m, 5), 3.78–3.60 (m, 8), 3.12 (m, 1), 2.85 (t, 2, J = 6.2 Hz), 2.84 (m, 2), 2.63 (q, 4, J = 7.1 Hz), 2.37 (dd, 2, J = 7.6 Hz), 1.68 (m, 4), 1.45 (m, 2), 1.07 (t, 6, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 173.82, 166.25, 163.96, 153.38, 142.92, 130.74, 124.55, 117.70, 70.49, 70.40, 69.30, 69.20, 64.11, 63.38, 63.26, 61.92, 60.27, 55.68, 51.11, 47.85, 40.49, 33.80, 28.31, 28.15, 24.75, 12.13; MS, m/z 638 (M^+), 429, 358. Anal. Calcd for $C_{30}H_{46}N_4O_9S$: C, 56.41; H, 7.26; N, 8.77; S, 5.02. Found: C, 56.23; H, 7.24.

1-Phenyl-2,5,8,11,14,17-hexaoxanonadecan-19-ol (H-6-Bn) (Scheme II, Compound 7b). Potassium *tert*-butoxide (5.12 g, 45.6 mmol) was added to hexaethylene glycol (12.34 g, 45.6 mmol) contained in a 100-mL round-bottomed flask equipped with a magnetic stirring bar and an inlet for argon. The resulting brown suspension became warm and was stirred for 15 min and then heated to 80 °C. Benzyl chloride (5.7 g, 45.0 mmol) was added dropwise over a period of 5 min. The reaction mixture turned dark brown and generated heat. The mixture was stirred for 10 h at 80 °C, then cooled to room temperature, and filtered. The viscous brown filtrate was chromatographed (silica gel 60, EtOAc) and yielded H-6-Bn (5.10 g, 30% yield) as a clear colorless oil: UV max (CH_3CN) 233 nm (ϵ 498); IR (thin film) 3495 (O—H), 2875 (C—H), 1460 (C=C), 1350 (C—O), 1110 (C—O), 740 (C=C), 700 (C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.35–7.27 (m, 5), 4.57 (s, 2), 3.71–3.59 (m, 24), 2.60 (br s, 1); ^{13}C NMR ($CDCl_3$) δ 138.26, 128.24, 127.61, 127.46, 73.12, 72.50, 70.54, 70.30, 69.41, 61.57; MS, m/z 373 ($M^+ + H^+$), 283, 175. Anal. Calcd for $C_{19}H_{32}O_7$: C, 61.27; H, 8.66. Found: C, 61.03; H, 8.81.

2-(Diethylamino)ethyl 4-[(1-Oxo-21-phenyl-2,5,8,11,14,17,20-heptaoxaheneicos-1-yl)amino]benzoate (P-6-Bn) (Scheme II, Compound 8b). Trichloromethyl chloroformate (1.01 g, 5.4 mmol) and benzene (5 mL) were placed in a 100-mL round-bottomed flask equipped with an addition funnel, magnetic stirring bar, and inlet for argon. The solution was cooled in an ice bath, and a solution of hexaethylene glycol monobenzyl ether (H-6-Bn) (1.84 g, 4.94 mmol) and *N*,*N*-dimethylaniline (0.66 g, 5.4 mmol) in benzene (10 mL) was added dropwise over a period of 5 min. The resulting white suspension was stirred at 4 °C for 12 h. The benzene was then removed quickly in vacuo and the residue cooled in an ice bath. A solution of procaine (1.17 g, 4.44 mmol) in pyridine (12 mL) was added in one portion. The resulting yellow suspension was stirred at 0 °C for 20 min and then at room temperature for 3.5 h. The reaction mixture was stirred with saturated $NaHCO_3$ solution (100 mL) and extracted with ethyl acetate (4 × 30 mL). The combined organic extracts were washed with saturated NaCl solution (1 × 30 mL), dried ($MgSO_4$), and concentrated in vacuo (85 °C, 0.1 torr) and yielded P-6-Bn (3.14 g, ca. 100% yield) as a yellow oil, which was nearly pure by TLC (silica gel, EtOAc/MeOH (1:1), R_f 0.55). This material was used in the next reaction without further purification. For analysis and testing, a sample was chromatographed (silica gel 60, EtOAc/MeOH (20:1)) to give P-6-Bn as a clear oil: UV max (95% EtOH) 270 nm (ϵ 5900); IR ($CHCl_3$) 3440 (N—H), 2950 (C—H), 1745 (urethane C=O), 1720 (ester C=O), 1615 (C=C), 1530 (C—O), 1418 (C—N), 1280 (C—O), 1180 (C—O), 1120 (C—O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.97 (d, 2, J = 8.7 Hz), 7.48 (d, 2, J = 8.7 Hz), 7.34–7.31 (m, 5), 4.54 (s, 2), 4.37–4.33 (m, 4), 3.75–3.59 (m, 22), 2.85 (t, 2, J = 6.3 Hz), 2.63 (q, 4, J = 7.1 Hz), 1.07 (t, 6, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 166.14, 153.08, 142.41, 130.86, 124.87, 117.56, 73.24, 70.61, 70.36, 69.44, 69.33, 64.44, 63.28, 61.73, 51.13, 47.87, 12.15; MS, m/z 634 (M^+), 395, 307. Anal. Calcd for $C_{33}H_{50}N_2O_{10}$: C, 62.44; H, 7.94; N, 4.41. Found: C, 62.29; H, 8.20; N, 4.47.

2-(Diethylamino)ethyl 4-[(19-Hydroxy-1-oxo-2,5,8,11,14,17-hexaoxanonadec-1-yl)amino]benzoate (P-6-H) (Scheme II, Compound 9b). Unpurified P-6-Bn (3.14 g, ca. 4.95 mmol), 10% Pd on activated carbon (0.612 g), absolute ethanol (45 mL), and 12 N HCl (0.5 mL) were placed in a Parr apparatus, and hydrogenation was allowed to proceed at 50 psi for 48 h. The reaction mixture was filtered and concentrated in vacuo. The residue was partitioned between ethyl acetate (100 mL) and saturated $NaHCO_3$ solution (50 mL). The aqueous layer was extracted with ethyl acetate (3 × 50 mL), and the combined organic extracts were washed with saturated NaCl solution (1 × 50 mL). The solution was dried ($MgSO_4$) and concentrated in vacuo. The residue was chromatographed (silica gel 60, EtOAc/MeOH (3:2)) to give P-6-H (2.40 g, ca. 89% yield) as a colorless oil: UV max (95% EtOH) 270 nm (ϵ 6000); IR ($CHCl_3$) 3450 (O—H), 2900 (C—H), 1750 (urethane C=O), 1720 (ester C=O), 1620 (C=C), 1530 (C—N), 1420 (C—O), 1280 (C—O), 1180 (C—O), 1120 (C—O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.97 (d, 2, J = 8.7 Hz), 7.88 (br s, 1), 7.47 (d, 2, J = 8.7 Hz), 4.39–4.31 (m, 4), 3.76–3.60 (m, 22), 2.85 (t, 2, J = 6.2 Hz), 2.63 (q, 4, J = 7.1 Hz), 1.07 (t, 6, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 166.18, 153.30, 142.77, 130.77, 124.57, 117.59, 72.56, 70.55, 70.47, 70.20, 70.08, 69.43, 69.29, 64.34, 63.16, 61.62, 61.52, 51.05, 47.77, 12.03; MS, m/z 544 (M^+). Anal. Calcd for $C_{26}H_{44}N_2O_{10}$: C, 57.34; H, 8.14. Found: C, 55.83; H, 8.81.

[3a*S*-(3aα,4β,6aα)]-19-[[4-[[2-(Diethylamino)ethoxy]carbonyl]phenyl]amino]-19-oxo-3,6,9,12,15,18-hexaoxanonadec-1-yl Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanoate (P-6-Bio) (Scheme II, Compound 10b). Biotin (0.314 g, 1.29 mmol), P-6-H (0.385 g, 0.7 mmol), dicyclohexylcarbodiimide (0.367 g, 1.78 mmol), 4-(*N*,*N*-dimethylamino)pyridine (0.036 g, 0.29 mmol), and methylene chloride (3 mL) were sealed, under an argon atmosphere, inside a 10-mL round-bottomed flask. The reaction mixture was sonicated at 40 °C for 72 h and then poured into ethyl acetate (50 mL) and filtered. The filtrate was concentrated in vacuo and the residue chromatographed (silica gel 60, EtOAc/MeOH (1:1)) to give P-6-Bio (0.525 g, 89% yield) as a white solid: mp 75–77 °C; UV max (95% EtOH) 270 (ϵ 28 500), 203 nm (28 500); IR ($CHCl_3$) 3480 (N—H), 3440 (N—H), 2975 (C—H), 1720 (C=O), 1610 (C=C), 1530 (C—N), 1280 (C—O), 1120 (C—O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.39 (br s, 1), 7.96 (d, 2, J = 8.7 Hz), 7.53 (d, 2, J = 8.7 Hz), 5.43 (br s, 1), 5.11 br s, 1), 4.54 (m, 1), 4.36 (t, 2, J = 6.2 Hz), 4.36–4.11 (m, 5), 3.76–3.51 (m, 20), 3.12 (m, 1), 2.84 (t, 2, J = 6.2 Hz), 2.83 (m, 2), 2.63 (q, J = 7.1 Hz), 2.37 (dd, 2, J = 7.6 Hz), 1.68 (m, 4), 1.45 (m, 2), 1.07 (t, 6, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 173.53, 166.22, 163.73, 153.51, 142.86, 130.79, 124.68, 117.70, 70.62, 70.52, 69.33, 69.17, 64.19, 63.41, 63.31, 61.98, 60.24, 55.52, 51.21, 47.88, 40.50, 33.84, 28.33, 28.28, 24.78, 12.19; MS, m/z 770 (M^+), 640. Anal. Calcd for $C_{36}H_{60}N_4O_{12}S$: C, 56.09; H, 7.58; N, 7.27. Found: C, 55.84; H, 7.60; N, 7.02.

[3a*S*-(3aα,4β,6aα)]-2-(Dimethylamino)ethyl 4-[[5-(Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]-

butylamino]benzoate (T-0-Bio) (Scheme II, Compound 1). Biotin (0.86 g, 3.5 mmol) and thionyl chloride (24.5 g, 207 mmol) were placed in a 100-mL round-bottomed flask equipped with a magnetic stirring bar and an inlet for argon. The biotin dissolved and gave a homogeneous yellow solution after being stirred for 15 min at room temperature. After an additional 15 min, the excess thionyl chloride was removed in vacuo. Dimethylformamide (DMF) (10 mL) was added to the residue, and the resulting solution was cooled to 0 °C. A solution of tetracaine (0.83 g, 3.15 mmol) and triethylamine (0.51 g, 50 mmol) in DMF (5 mL) was added dropwise, giving a cloudy mixture, which was stirred at 0 °C for 1 h and then at 25 °C for 1 h. The reaction mixture was poured into saturated $NaHCO_3$ solution (100 mL) and extracted with EtOAc (4 × 30 mL). The combined organic extracts were washed with saturated NaCl solution (1 × 200 mL), dried ($MgSO_4$), and concentrated in vacuo to give a yellow oil (1.3 g). This material was chromatographed (silica gel 60, EtOAc/MeOH (3:2)) and gave, after isolation, 1.05 g (68% yield) of T-0-Bio as a white solid: UV max (95% EtOH) 270 (ϵ 20 000), 203 nm (16 000); IR ($CHCl_3$) 2940 (CH), 1705 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.12 (d, 2, J = 8.7 Hz), 7.22 (d, 2, J = 8.7 Hz), 5.14 (br s, 1), 4.82 (br s, 1), 4.50 (m, 3), 4.27 (m, 1), 3.67 (t, 2, J = 7.4 Hz), 3.09 (m, 1), 2.9 (m, 3), 2.7 (m, 1), 2.49 (s, 6), 2.1–1.1 (m, 12), 0.8 (dd, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 171.98, 165.44, 163.12, 147.08, 131.21, 129.22, 128.20, 61.86, 60.09, 57.30, 55.23, 48.99, 45.07, 40.38, 33.88, 29.96, 28.18, 25.21, 19.96, 13.63; MS, m/z 490 (M^+). Anal. Calcd for $C_{25}H_{38}N_4O_4S$: C, 61.20; H, 7.81. Found: C, 61.29; H, 7.62.

Avidin Purification. Due to the high potassium salt content of commercial, affinity-purified avidin (Sigma Chemical Co., St. Louis, MO), all avidin stocks were dialyzed with 0.5 M ammonium acetate across standard dialysis tubing (MW cutoff 3500; Spectvapor 3, Spectrum Medical Industries, Inc., Los Angeles, CA) and then lyophilized.

All avidin concentrations were expressed in terms of the concentration of biotin-binding sites. Avidin normally exists as a cylindrical tetramer with four identical biotin-binding sites, two on each face.[10] Therefore, the actual concentration of avidin tetramers would be one-fourth the avidin concentrations that are given in this paper.

Electrophysiology. Adult frogs (*Rana pipiens*, purchased from Connecticut Valley Farms, Northampton, MA) of either sex and measuring from 3 to 3.5 in. in length from snout to rump were pithed. Each sciatic nerve was exposed from its origin in the lumbar plexus to its bifurcation at the knee and removed. The excised nerves were refrigerated (15 °C) in frog Ringer solution for up to 5 days before use, previous data having demonstrated no physiological changes attendant to such storage.[15]

In preparation for these electrophysiological studies, the previously excised sciatic nerves were desheathed, split in half lengthwise, and mounted in a sucrose gap chamber similar to that described by Strong et al.[16] The Ringer solution used in these studies contained NaCl, 110 mM; $CaCl_2$, 2.0 mM; KCl, 2.5 mM; and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES)–HCl buffer, 5.0 mM, at a pH of 7.20 ± 0.02. In some pilot experiments, tetraethylammonium chloride (TEA), 5 mM, was included in the Ringer solution to block potassium channels. Isotonic sucrose solution (0.18 M) flowed at 2–3 mL/min through the central partition of the nerve chamber in most experiments. The potency of local anesthetics and their derivatives for impulse blockade was unaffected by the TEA, but was decreased in the presence of sucrose, as previously reported by Courtney et al.[17,18] All dose–response data were calculated when sucrose, but no TEA, was present. All chemicals were reagent grade or better.

Supramaximal cathodal stimuli (50-μs duration) were applied by using a World Precision Instruments Model 850 stimulator (New Haven, CT). The evoked action potentials were amplified, filtered (differential electrometer amplifier model AK4FUU, MetaMetrics, Carlisle, MA), and then displayed on a digital storage oscilloscope (Nicolet Instrument Corp., Madison, WI). Analog recordings were plotted on a Watanabe (Irvine, CA) Model WX4421 plotter. Base-line recording in Ringer solution continued for at least 20 min to ensure that a steady state had been achieved before a drug was added. Solution in the test chamber was then exchanged for Ringer solution containing a test drug, avidin, or both. Due to their low solubility in aqueous media, all local anesthetic derivatives were dissolved initially in dimethyl sulfoxide (Me_2SO). Aliquots of this stock were diluted with Ringer solution and then applied to the nerves. Me_2SO formed 5% (vol/vol) or less of the final drug solution and, by itself, had no effect on impulse activity. The height of the action potential was recorded for at least 20 min following each change of solution.

"Tonic" block was calculated as the percent decrement in the action potential height produced by a given treatment, relative to height of the control action potential elicited by infrequent stimulation (<0.5 min^{-1}). "Use-dependent" block was defined as any additional reduction in height of the action potential that resulted from an increase in stimulation frequency from <0.5 to ≤40 Hz, expressed as percent of the height of the control action potential. "Use-dependent" block was measured in the presence of a steady-state "tonic" block of between 30% and 70%. "Tonic" EC_{50}'s (the drug concentration that reduced impulse height by 50%) were calculated by interpolation using drug concentrations that produced (on the average) 20–80% inhibition of the action potential. The generated log dose–response curve (d–r) is nearly linear in this range. A standard linear regression program was used to calculate the EC_{50}'s and the correlation coefficients for d–r data. "Leash" lengths (defined as the distance between the terminal tertiary amine nitrogen of the anesthetic and the surface of the avidin molecule) for the various local anesthetic derivatives were calculated by using standard values for bond lengths and the known depth of the biotin-binding site beneath the surface of avidin.[10,19]

Table I. Potency of Local Anesthetics and Derivatives for Tonic Inhibition of Compound Action Potential

local anesthetic[a]	EC_{50},[b] M	no. of determinations[c]	no. of nerves[d]	corr coeff[e]
procaine	1.3×10^{-3}	21	5	0.88
P-0-Bio[e]	4.2×10^{-3}	14	8	0.73
P-3-Bio	2.4×10^{-3}	9	6	0.74
P-6-Bio	4.5×10^{-3}	22	12	0.60
P-3	3.2×10^{-3}	13	5	0.73
P-6	6.6×10^{-3}	9	5	0.71
tetracaine	1.2×10^{-5}	9	4	0.73
T-0-Bio	1.5×10^{-3}	7	3	0.79

[a] See Figure 1 for abbreviation scheme. [b] Concentration for 50% reduction of infrequently stimulated compound action potential. See text for method of calculation. [c] Number of data points used to generate regression line. [d] Number of nerves used to determine data points. [e] Calculated for nearly linear portion of log dose–response curve between 20% and 80% of maximal response.

Results

Absence of Avidin. All local anesthetics and local anesthetic derivatives produced dose-dependent, reversible inhibition of the action potential when applied in the absence of avidin. The order of potency observed was tetracaine > procaine > T-0-Bio > P-3-Bio > P-3 > P-0-Bio > P-6-Bio > P-6 (Table I). Detailed dose–response data are given in Figure 1.

The time required to reach steady-state "tonic" inhibition of the action potential after drug application was nearly 20 min for procaine and tetracaine and their various derivatives (Figure 2). Approximately 6.5–7 min was required to achieve 50% of steady-state inhibition of action potentials produced by equipotent concentrations of tetracaine (20 μM) and T-0-Bio (1 mM) (Figure 2). No ad-

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(16) Strong, P. N.; Smith, J.; Keana, J. F. W. *Toxicon* **1973**, *11*, 433.
(17) Courtney, K. R.; Kendig, J. J.; Cohen, E. N. *Anesthesiology* **1978**, *48*, 111.
(18) Courtney, K. R. *J. Pharmacol. Exp. Ther.* **1980**, *213*, 114.
(19) *Tables of Interatomic Distances and Configuration in Molecules and Ions*; Sutton, L. E., Ed.; The Chemical Society: London, 1958.

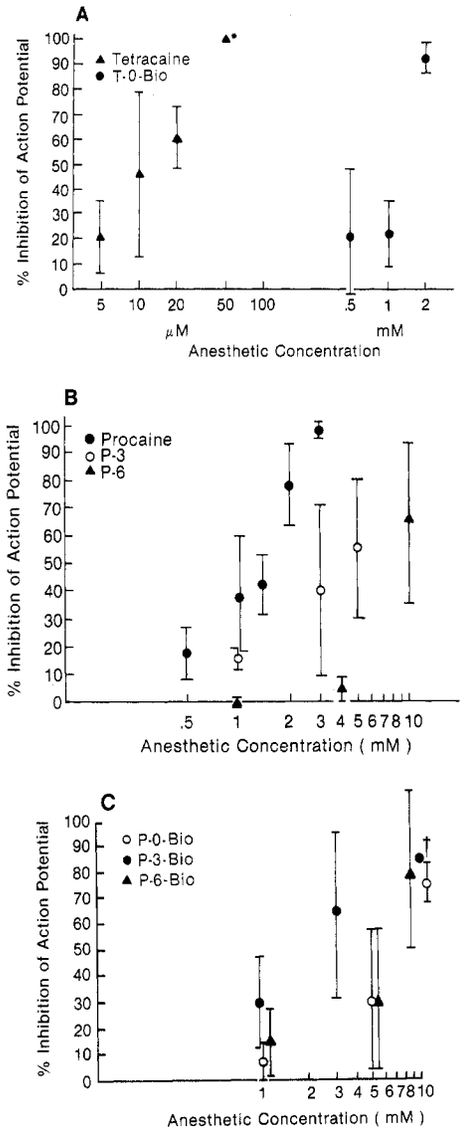

Figure 1. Steady-state block of compound action potentials by local anesthetics and derivatives: (A) tetracaine and T-0-Bio, (B) procaine and nonbiotinylated derivatives, (C) biotinylated procaine derivatives. All data expressed as means ± SD of four or more (except where indicated) determinations under sucrose gap conditions; (*) single determination, (†) two determinations.

vantage was observed whenever the recording was continued for longer than 20 min. Figure 2 also documents the reversible nature of the block by both parent compounds and derivatives.

Biotinylated derivatives produced less use-dependent block of the compound action potential than did the parent local anesthetics at comparable levels of steady-state "tonic" block (Figure 3), and they also reached that block at a slower rate than the parent drugs. For example, procaine required 3 pulses at 40 Hz whereas P-0-Bio, P-3-Bio, and P-6-Bio required 15, 9, and 14 pulses, respectively, to reach *half* of the additional use-dependent block that would ultimately be present at steady state.

Presence of Avidin. Dialyzed avidin by itself had no effect on action potentials when applied in concentrations up to 20 mM. All biotinylated local anesthetic derivatives lost their ability to produce both "tonic" and "use-dependent" decrements in action potential height when applied with equimolar avidin. Scheme I illustrates how avidin binding determines the depth of membrane penetration by biotinylated ("leashed") local anesthetic derivatives. Figure 4 shows a representative experiment with

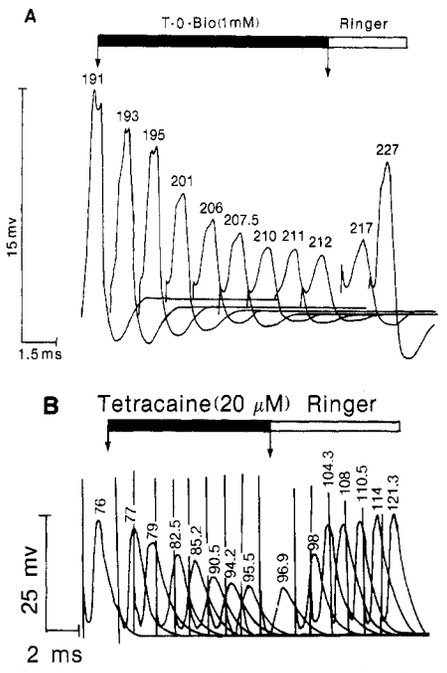

Figure 2. Typical onset and offset of block of action potentials by T-0-Bio (A) and tetracaine (B). Biphasic action potentials are present in (A) because sucrose gap conditions were not used as in (B). Onset and offset times were unchanged by sucrose gap conditions. Numbers above traces indicate experimental time in minutes.

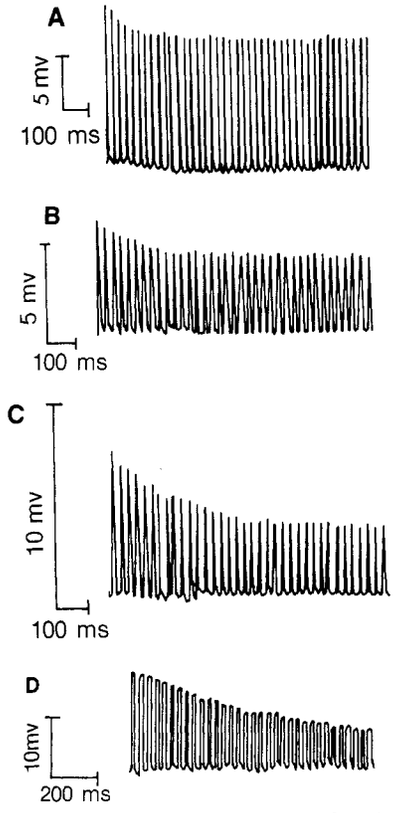

Figure 3. Use-dependent block of compound action potentials by local anesthetics and derivatives. Supramaximal stimuli were applied at 40 Hz to desheathed nerves under sucrose gap conditions. (A) Procaine, 1.5 mM, 50% tonic block; (B) P-0-Bio, 10 mM, 65% tonic block; (C) P-3-Bio, 10 mM, 65% tonic block; (D) P-6-Bio, 5 mM, 50% tonic block.

T-0-Bio in which avidin has prevented the local anesthetic derivative from producing nerve block.

We wondered whether avidin, containing four biotin-binding sites, might be inhibiting the action of the local

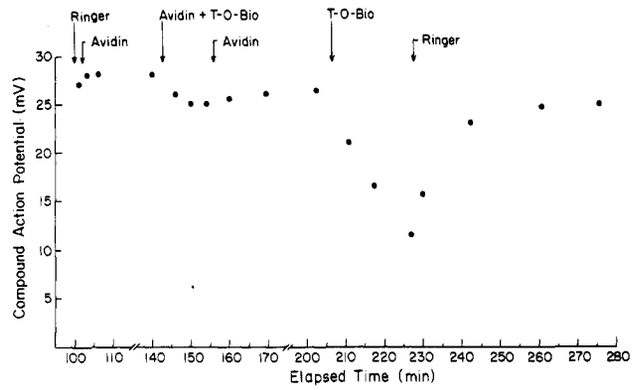

Figure 4. T-0-Bio (0.5 mM) induced inhibition of compound action potentials (in the presence and absence of) equimolar avidin. Sucrose gap conditions were not used in this instance.

anesthetic derivatives by binding and orienting two or three of every four derivative molecules in a direction from which the sodium channel could not be reached. We tested for this possibility by incubating nerves with quadruple the calculated EC_{50} and sufficient avidin to fully bind the local anesthetic derivative. Nerve block also was prevented under these conditions, making "sequestration" of anesthetic an unlikely mechanism for the abolition of anesthetic-induced nerve block by avidin.

On the basis of these findings and the calculated "length" of our longest derivative (P-6-Bio), we concluded that the local anesthetic binding site is located deeper than ca. 15 Å from the external surface of the neuronal plasma membrane. The precise depth is difficult to define, due to uncertainties about the conformation of the "leashed" local anesthetic and the accompanying uncertainty about the mean anesthetic–biotin distance.

Discussion

In this study, the addition of polyethylene glycols and biotin to the aromatic amino terminus of procaine produced compounds retaining tonic impulse-blocking activity, albeit with less potency than the parent drugs. The derivatives produced use-dependent block at stimulation frequencies comparable to those required for native procaine; they required, however, a greater number of pulses than procaine to achieve smaller steady-state inhibition, even though the derivatives were always present at 2–4-fold-higher concentrations. Adding biotin to the aromatic secondary amine of tetracaine produced a compound with greatly decreased potency for tonic impulse blockade. Use-dependent block was slower in onset, similar to that of the biotin-substituted procaine derivatives. No biotin-containing local anesthetic derivative blocked nerve impulses when equimolar avidin was present.

The markedly reduced potency of biotin-substituted tetracaines contrasts with the minimally reduced potency of biotinyl procaines. We explain these differences by noting the size and solubility characteristics of polyethylene glycols and biotin relative to the parent local anesthetics.[9,20] The 50–100-fold-greater potency of tetracaine relative to procaine is often attributed to greater lipid solubility resulting from butyl substitution of the aromatic amine.[20] The only other difference between tetracaine and procaine, the diethyl vs. dimethyl substitution of the tertiary amino terminus, has little effect on anesthetic potency.[21] Because of their limited lipid solubility, adding polyethylene glycols or biotin to tetracaine would be more likely to produce greater changes than a similar modification of procaine. P-0-Bio is considerably bulkier than procaine, but should have comparable lipid solubility. T-0-Bio, on the other hand, has physicochemical features resembling P-0-Bio more than the parent anesthetic, tetracaine. Where tetracaine has a single, highly lipid-soluble alkyl substituent, T-0-Bio has an additional bulky, amphophilic group, much larger than the nearby butyl chain. This amphophilic group may interfere with the normal association of the butyl moiety and membrane lipids or hydrophobic regions of the sodium channel.

The identity and purity of our synthetic products were tested by the usual methods. Their stability was not tested directly, but there was no evidence of a decrement in their potency during storage over 1 year at −20 °C.

Unless the affinity of avidin for biotinylated local anesthetics is sufficiently great that a nearly irreversible association results, any conclusions drawn regarding the depth of membrane penetration of local anesthetic derivatives will be in error. Fortunately, the K_a for the avidin–D-biotin interaction is roughly 10^{15} M^{-1}, and Romovacek et al. found that substitution of ACTH on the carboxyl tail of D-biotin caused insignificant reductions in the affinity of avidin for the substituted biotin.[8,11] Similarly, Lobel et al. measured high affinity binding of biotinylated cobra toxin to avidin.[13] Biotinylated insulin, on the other hand, bound succinoylavidin with considerably reduced affinity relative to biotin itself, reducing the half-time for dissociation from 127 days to roughly 1 h, and thus increasing the off-rate from 3.2×10^{-7} s^{-1} to 3.8×10^{5} s^{-1}. The stability and half-time for dissociation increased when a spacer molecule, similar to the polyethylene glycols we used, was interposed between biotin and insulin. On the basis of those findings, then, we presume that the association of avidin with derivatized local anesthetic is of comparably high affinity at least for local anesthetic derivatives containing polyethylene glycol spacers (such as P-6-Bio). More likely, all local anesthetic-biotin derivatives bind avidin with high affinity, in common with the freely mobile, sterically unhindered biotinyl-ACTH. We predict that the on-rate for avidin–biotinylated local anesthetic association will be diffusion-limited and hence will be roughly 10^{9} M^{-1} s^{-1}.[22] The off-rate should then be around 10^{-6} s^{-1}. During an experiment with avidin-bound local anesthetic derivative, the unbound derivative concentration should rise no higher than 6 μM, a concentration 100-fold below that which produced measurable impulse blockade (Figure 1A).

Similarly, in an attempt to define the depth of the D-biotin-binding site on avidin, Green et al. studied a series of bisbiotin-substituted diaminoalkanes of increasing carbon chain length.[10] They allowed these bifunctional compounds to associate with avidin and then examined them with electron microscopy. They found that the likelihood of both biotins being bound by avidin increased in association with increasing chain length, up to chains of 12 carbon atoms. Longer alkanes had no greater likelihood of bifunctional binding, indicating that some sort of length-related steric hindrance had been overcome. Thus, Green et al. presumed that 12 carbon atoms provided the minimal distance necessary for avidin–biotin association at both ends of the alkane. This would place

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the carboxyl end of biotin between 6 and 9 Å beneath the surface of avidin. On the basis of these values and using a molecular model (CPK series, Ealing Co., Natick, MA), we estimate a distance of 15–18 Å from the tertiary amine nitrogen of P-6-Bio to the surface of avidin. In terms of planar lipid bilayer architecture, this depth extends well beyond the phospholipid head groups and into the region of the acyl chains.[23]

Finn et al. showed that avidin-bound, biotinylated ACTH and similarly conjugated insulin could bind to cell-surface receptors and produce their usual biochemical effects, even in the absence of a spacer molecule between biotin and the hormone.[12] Thus we feel that our local anesthetic derivatives were sufficiently long to permit concurrent binding to avidin and to a receptor on the cell surface (if one were present). The lack of potency of avidin-bound, biotinylated local anesthetics provides further evidence that there is no local anesthetic binding site on or near the external membrane surface, a result in common with that deduced from external application of quaternary, permanently charged lidocaine derivatives, which also were inactive.[2,3] Perhaps, like these quaternary drugs, our avidin-bound, biotinylated local anesthetics might produce nerve block if applied on the cytosolic side of a nerve membrane.[2,3]

Reduction in the action potential elicited in desheathed frog sciatic nerve is not a definitive method for assaying local anesthetic mechanisms. It was, however, an appropriate assay for this investigation since our intent had been to measure local anesthetic potency rather than detailed interactions with the sodium channel.

We recognize that the reduced onset rate and efficacy of our local anesthetic derivatives in producing "use-dependent" block may have resulted from the increased molecular weight, from a change in lipid solubility, or from the decreased mobility attendant to the increased size of the compounds,[18] rather than from decreased affinity for the sodium channel as a result of our chemical modifications. Our data were insufficient to test these alternative explanations. Current theories for the mechanism of "use-dependent" block in nerve support drug binding to the "open" state of the sodium channel as the primary integral event.[24] An increased lipid solubility might favor diffusion through the lipid bilayer and might disfavor diffusion through the aqueous pore of the channel to an anesthetic binding site located therein.

We assume that the membrane permeability of the "anesthetic portion" of local anesthetic derivatives is not substantially different from that of the "parent" local anesthetics. We tested this assumption, albeit indirectly, by treating nerves with local anesthetic derivatives in the absence of avidin. All local anesthetic derivatives diffused into the nerve bundle and reached their site of action without unusual delay, producing steady-state inhibition of the action potential with rates of onset comparable to those of the parent local anesthetics.

We assume that there is no repulsion between the surface of the plasma membrane and avidin. Avidin is a basic protein with an isoelectric point near pH 10.5;[8] at physiologic pH, avidin carries a net positive charge, which should provide for electrostatic attraction of avidin to the usually negatively charged neuronal membranes.[25]

Finally, and perhaps most importantly, we assume that tertiary amine local anesthetics may reach their binding site via a hydrophobic pathway. Should this assumption prove to be incorrect, our depth measurement data may be used only to exclude a cell-surface receptor.

In summary, our data support two conclusions: First, we could not detect an anesthetic receptor within 15 Å of the external surface of the neuronal plasmalemma; second, local anesthetics conjugated to polyethylene glycols and biotin through their *p*-amino nitrogens retained activity in the absence of equimolar avidin, implying that conservation of the molecular conformation of this region of the molecule may not be as important to potency as conformation of other regions. We predict that further experiments in which our anesthetic derivatives are applied within the cytoplasm and in which newer products with longer "leashes" are applied to the external surface of the plasmalemma may more completely identify the locus of local anesthetic binding within the sodium channel. Finally, these chemical modifications make antagonism of local anesthetic action a theoretical possibility.

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