Proteolytic Nicking of the Acetylcholine Receptor†

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ABSTRACT: Low concentrations of papain rapidly cleave solubilized or membrane-bound acetylcholine receptor (AcChR) from *Torpedo californica* into a wide range of small fragments. The α subunits of the receptor are most resistant to cleavage. After solubilization in sodium dodecyl sulfate solutions the fragments are dissociated, and on electrophoresis the apparent subunit composition is reduced from four types (α, β, γ, and δ) to only α and finally, with large amounts of papain, to fragments even smaller than α. Prior to dissociation in sodium dodecyl sulfate, the proteolytic fragments remain physically and functionally associated. Thus, receptor which has been degraded so as to apparently contain only α subunits, or even no obvious subunits, still retains antigenic determinants corresponding to each subunit, still retains its characteristic size and doughnut shape when examined electron microscopically, and still sediments as dimers on sucrose gradients. Moreover, proteolytically nicked receptor remains fully functional in carbamylcholine-induced $^{22}Na^+$ flux. These results demonstrate that inadequate inhibition of proteases during purification of receptor could account for reports from some laboratories that they have purified receptors containing only α subunits or fragments of α subunits. Also, our results demonstrate the strong noncovalent association between AcChR subunits which has thus far precluded their separation except under denaturing conditions in sodium dodecyl sulfate.

Acetylcholine receptor (AcChR)[1] from *Torpedo californica* is composed of four subunits designated α, β, γ, and δ (Weill et al., 1974; Raftery et al., 1975; Lindstrom et al., 1978; Hucho et al., 1978; Froehner & Rafto, 1979). These subunits are present in AcChR monomers in the mole ratio $\alpha_2\beta\gamma\delta$ (Damle & Karlin, 1978; Reynolds & Karlin, 1978; Lindstrom et al., 1979b). Corresponding subunits have been identified in other species of *Torpedo* (Claudio & Raftery, 1977) and in AcChR from *Electrophorus electricus* (Lindstrom et al., 1980a). Four corresponding sets of antigenic determinants have been detected in AcChR from muscle (Lindstrom et al., 1978; 1979a,b), and muscle AcChRs composed of several types of subunits have been purified (Boulter & Patrick, 1977; Nathanson & Hall, 1979; Lindstrom et al., unpublished results). The α subunits of all these AcChRs have apparent molecular weights of 38 000–42 000, depending on the species, and are known to contain part or all of the acetylcholine binding site (Karlin et al., 1976). It is known that the cation channel regulated by acetylcholine binding is an integral component of the AcChR monomer (Wu & Raftery, 1979; Changeux et al., 1979; Anholt et al., 1980; Lindstrom et al., 1980a). The functions of β, γ, and δ are unknown, but it is suspected that one or more might constitute the cation channel.

There are persistent reports from some laboratories that AcChR consisting only of α subunits can be purified from *Torpedo* electric organ (Sobel et al., 1977) or mammalian muscle (Shorr et al., 1978; Merlie et al., 1978). The observation that these preparations have the same sedimentation coefficient on sucrose gradients as AcChR preparations containing four subunits and that they have the same specific activity for binding [^{125}I]-α-bungarotoxin ([^{125}I]-α-BGT) is inconsistent with the interpretation that they consist entirely of α subunits. It has recently been reported that *Torpedo* AcChR containing no γ subunit can be purified and reconstituted (Huganir et al., 1979). All of these preparations have in common, by contrast with the preparations found to contain four subunits, the fact that they were prepared without the use of EDTA and iodoacetamide as protease inhibitors in the case of electric organ AcChRs and a complex additional array of protease inhibitors in the case of muscle AcChRs. It has also been reported that trypsin-treated AcChR can be prepared which contains only a 27 000 molecular weight fragment of the α subunit, yet which sediments at nearly the same rate as intact monomers (Bartfield & Fuchs, 1979).

Some effects of proteases on AcChR structure and function have been previously reported. Trypsinization of *Electrophorus* AcChR simplifies its gel electrophoresis pattern (Lindstrom et al., 1976), while only reducing its apparent size on sucrose gradient sedimentation by 0.5 S. Trypsinization of *Torpedo* AcChR also cleaves the dimers normally observed by sucrose gradient sedimentation in this species to monomers of the size typically observed in other species (Lindstrom, 1976).

Here we show that the apparent subunit structure of AcChR is very protease sensitive. The more protease-sensitive higher molecular weight subunits can easily be nicked into an array of small fragments, with the result that only the α subunit is apparent after dissociation in sodium dodecyl sulfate (NaDodSO$_4$) and electrophoresis on acrylamide gels. We show that the proteolytic fragments remain tightly associated and that extensive proteolysis does not impair either toxin binding or cation channel function. These results suggest that the failure of some laboratories to recognize the presence of the higher molecular weight subunits in AcChR may result from insufficient prevention of proteolysis. Our results also emphasize the strong noncovalent association between AcChR subunits which has thus far precluded their separation except under denaturing conditions in NaDodSO$_4$.

Materials and Methods

Soluble *T. californica* (Pacific Biomarine) AcChR was affinity purified on toxin–agarose (Lindstrom et al., 1978). AcChR subunits were purified by preparative electrophoresis and used to raise antisubunit sera in rats (Lindstrom et al., 1978, 1979a,b). Electrophoresis was conducted as previously described (Lindstrom et al., 1978).

AcChR in membranes was purified by discontinuous and continuous sucrose gradient centrifugation, as described in

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[1] Abbreviations used: AcChR, acetylcholine receptor; NaDodSO$_4$, sodium dodecyl sulfate; [^{125}I]-α-BGT, ^{125}I-labeled α-bungarotoxin.

0006-2960/80/0419-4791$01.00/0

detail elsewhere (Lindstrom et al., 1980a), using methods similar to those reported by others (Hamilton et al., 1977; Reed et al., 1975). The alkaline extraction method of Neubig (Neubig et al., 1979) was used in a similar manner to that described by others (Wu & Raftery, 1979; Changeux et al., 1979) to prepare purified membrane-bound AcChR. These vesicles were reconstituted into model lipid vesicles by using the method of Epstein & Racker (1978), as described in detail elsewhere (Lindstrom et al., 1980a) and essentially as described by Wu & Raftery (1979) and Changeux et al. (1979). The reconstituted vesicles contained 4.63×10^{-7} M [^{125}I]-α-BGT binding sites and 25 mg/mL soybean lipid.

Reconstituted vesicles were treated with papain (Sigma 2× crystallized) as follows. Aliquots (200 μL) of vesicles were added to tubes containing 5 μL of papain solutions giving final concentrations of 0.1–0.8 μg/mL. After 5 min, 6.7 μL of 30 mM iodoacetamide was added to each tube to inhibit the papain. Soluble AcChRs (4.25 mg/mL, specific activity 9.2 nmol of [^{125}I]-α-BGT binding sites/mg of protein) were digested with papain as follows. Aliquots (10 μL) of AcChR were added to tubes containing 10 μL of papain solutions, giving final concentrations of $0.47–3.8 \times 10^{-3}$ μg of papain/μg of AcChR. After 5 min, 10 μL of 30 mM iodoacetamide was added.

Carbamylcholine-induced $^{22}Na^+$ influx into vesicles containing reconstituted AcChR was measured by the method of Epstein & Racker (1978). Briefly, tubes containing 5 μL of 0.2 mCi/mL $^{22}Na^+$ (NEN) and 5 μL of 10^{-3} M carbamylcholine (Sigma) or 5 μL of water were prepared in triplicate. Reconstituted vesicles (40 μL in 145 mM sucrose, 10 mM sodium phosphate buffer, pH 7.5, and 5 mM NaN_3) were added, mixed, and within 30 s applied to 2-mL columns of Dowex 50 X8-100 (Sigma, Tris form, in water, washed with 3 mL of 3 mg/mL bovine serum albumin in 170 mM sucrose). The columns were eluted with 3 mL of 175 mM sucrose, and the $^{22}Na^+$ in the eluate was measured in a γ counter. Carbamylcholine induces influx of $^{22}Na^+$, which is measured as the excess of $^{22}Na^+$ in carbamylcholine-treated samples over water blanks. In reconstituted vesicles carbamylcholine at 10^{-4} M induces maximal influx of ^{22}Na, and influx is proportional to the amount of active AcChR (Lindstrom et al., 1980a).

Radioimmune assays were conducted essentially as previously described (Lindstrom et al., 1978). Papain-treated soluble AcChR aliquots were diluted to 1 mL with a solution containing 0.5% Triton X-100, 100 mM NaCl, 10 mM sodium phosphate, pH 7.5, and 10 mM NaN_3. Aliquots (60 μL) were mixed with excess [^{125}I]-α-BGT (22.8 μL of 2×10^{-6} M) and then diluted to 22.8 mL in the Triton X-100 buffer. In triplicate, 1-mL aliquots were incubated overnight with 5 μL of normal rat serum, antiserum to native *Torpedo* AcChR, or antiserum to α, β, γ, or δ. This provided excess anti-AcChR over AcChR (antisera titers in moles of [^{125}I]-α-BGT binding sites of AcChR bound/liter of serum: α, 7.5×10^{-6} M; β, 4.8×10^{-6} M; γ, 5.3×10^{-6} M; δ, 4.8×10^{-6} M).

Sedimentation behavior of the papain-treated AcChR was investigated by using linear sucrose gradients (5 mL, 5–20% sucrose in 0.5% Triton X-100, 100 mM NaCl, and 10 mM sodium phosphate buffer, pH 9.5). Gradients were layered with 50 μL of samples and centrifuged at 50 000 rpm in a Beckman SW 50.1 rotor for 6 h at 5 °C. Fractions of gradients were collected from the bottom of the tube.

Morphology of both the native and the papain-treated AcChR was studied by the negative staining technique. Solubilized AcChR at a concentration of 100 μg/mL [in 0.2 M sodium bicarbonate, pH 9.5, 0.1% Triton X-100, and 10 μg/mL bacitracin (to improve the spreading of samples)] was layered on a carbon-coated copper grid, washed with 0.1% ammonium acetate, stained with 1% uranyl acetate, and dried. The specimens were examined with a JEOL 100B or 100 CX electron microscope, operating at 80 kV and fitted with a 100-μm objective aperture. Electron micrographs were taken at 100 000× direct magnification and subsequently enlarged with a point source illumination enlarger.

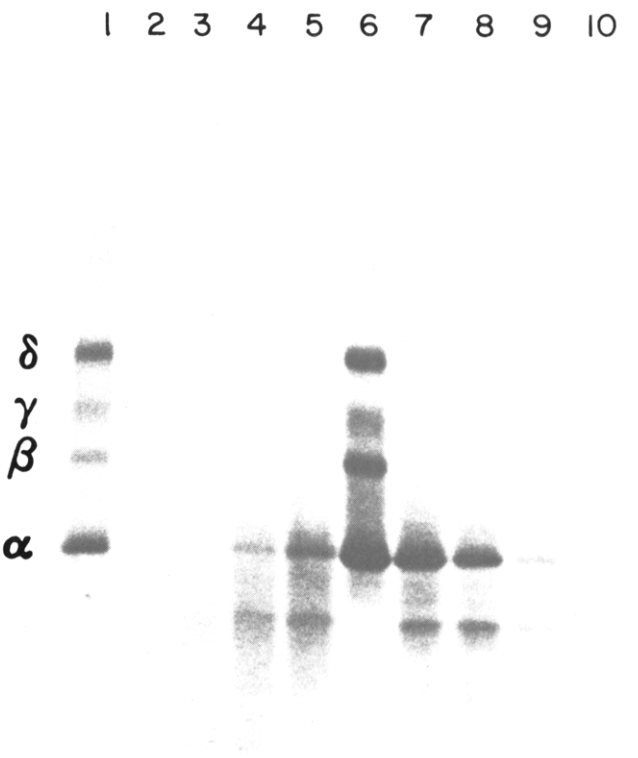

FIGURE 1: Acrylamide gel electrophoresis of membrane-bound and soluble AcChR after proteolysis with papain. (1) AcChR reconstituted into soybean lipid vesicles (20-μL aliquot). (2–5) AcChR in vesicles after treatment for 5 min with 0.8, 0.4, 0.2, and 0.1 μg/mL papain, respectively (20-μL aliquots). These are the same vesicles that were used in Table II. (6) Solubilized AcChR (5 μg). (7–10) Solubilized AcChR after treatment for 5 min with 1, 2, 4, and 8 μg/mL papain, respectively (5 μg). These are the same preparations that were used in Table I.

Results

The pattern of bands characteristic of the subunits of solubilized and purified AcChR after acrylamide gel electrophoresis in $NaDodSO_4$ is greatly affected by treatment of AcChR with very small amounts of papain. A 5-min exposure to even 5×10^{-5} μg of papain/μg of AcChR removes all traces of the higher molecular weight subunits β, γ, and δ (data not shown). Figure 1 (6–10) illustrates an experiment using much higher amounts of papain. After exposure to 4.7×10^{-4} μg of papain/μg of AcChR for 5 min, all traces of β, γ, and δ are eliminated and some traces of a proteolytic fragment are visible at a lower apparent molecular weight than α (Figure 1, 7). From studies of papain treatment of purified α (W. Gullick and J. Lindstrom, unpublished results), we know this to be a fragment of α. After exposure to 3.8×10^{-3} μg of papain/μg of AcChR for 5 min, no trace of the intact α subunit remains (Figure 1, 10).

We investigated whether proteolytic nicking of the subunits caused dissociation of peptide fragments from the macromolecular complex. AcChR treated with 3.8×10^{-3} μg of papain/μg of AcChR and then labeled with [^{125}I]-α-BGT sedimented on sucrose gradients identically with untreated

Table I: Effect of Proteolysis on Immune Precipitation

antiserum	% of [^{125}I]-α-BGT-labeled AcChR immune pptd by antiserum to native AcChR				
	0 μg of papain	1 μg of papain	2 μg of papain	4 μg of papain	8 μg of papain
anti-α	108	102	110	101	105
anti-β	111	101	107	101	101
anti-γ	101	95	103	94	102
anti-δ	93	90	95	88	92

Table II: Effect of Proteolysis on $^{22}Na^+$ Influx Induced by 10^{-4} M Carbamylcholine

sample	carbamylcholine-induced $^{22}Na^+$ influx (cpm ± SE)	% initial response
reconstituted vesicles	1548 ± 118	100
reconstituted vesicles + 0.1 μg/mL papain	1503 ± 126	97
reconstituted vesicles + 0.2 μg/mL papain	1361 ± 86	88
reconstituted vesicles + 0.4 μg/mL papain	1348 ± 80	87
reconstituted vesicles + 0.8 μg/mL papain	1336 ± 58	86

AcChR, showing no conversion of dimers to monomers or decrease in the apparent size of the monomers (data not shown). Huganir & Racker (1980) have obtained a similar result. This result suggested that papain cleaved off no large portion of the AcChR molecule. We then used immunoprecipitation to investigate whether any subunit was dissociated from the macromolecular complex by proteolysis. Proteolyzed AcChR was labeled with [^{125}I]-α-BGT and immune precipitated with an excess of antisera to native AcChR and to each subunit. Table I shows that even the most extensive proteolysis employed produced no inhibition of [^{125}I]-α-BGT binding and that all AcChR molecules retained the antigenic determinants of each subunit, even though only the α or no intact subunits at all remained.

Electron microscopy of negatively stained solubilized AcChR and papain-treated AcChR showed that after a 5-min exposure to 3.8×10^{-3} μg of papain/μg of AcChR, a treatment sufficient to cleave all α, β, γ, and δ subunits, the AcChR monomers retained their characteristic doughnut shape (Cartaud et al., 1973, 1978; Nickel & Potter, 1973; Orci et al., 1974; Allen & Potter, 1977; Ross et al., 1977; Heuser & Salpeter, 1979) (Figure 2). AcChR dimers were observed after proteolysis, as expected from the sucrose gradient sedimentation experiment.

In order to test whether proteolyzed AcChR retained ion channel function in addition to ligand binding, the antigenic structure of each subunit, dimeric organization, and native molecular shape, we reconstituted purified AcChR into vesicles of soybean lipid. Treatment with papain produced the same pattern of degradation as that observed with detergent-solubilized AcChR. Thus, the membrane did not protect any part of the AcChR from access to papain. Table II shows that even the most extensively proteolyzed samples showed only slight inhibition of carbamylcholine-induced $^{22}Na^+$ flux.

Discussion

These results clearly show that proteolysis can cleave AcChR subunits into an array of fragments which may obscure the recognition that the subunits are components of the macromolecule. Apparent loss of β, γ, and δ due to their greater protease sensitivity probably accounts for reports from several laboratories that AcChR can be prepared consisting

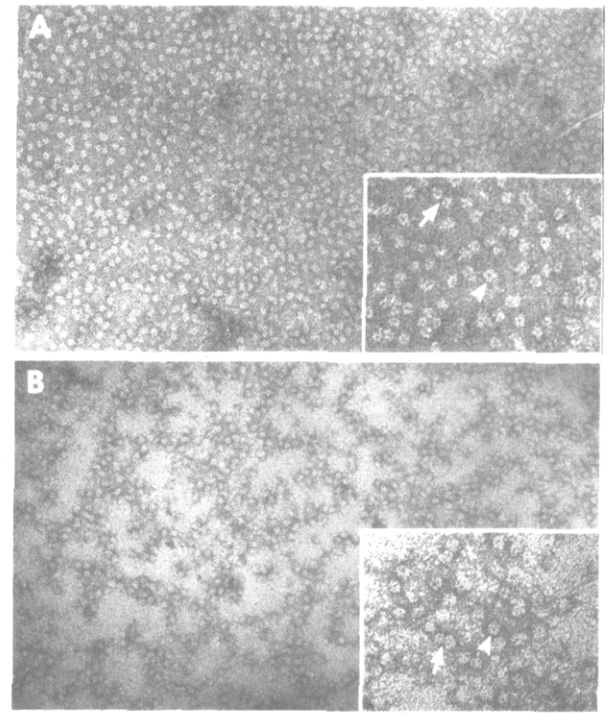

FIGURE 2: Electron micrograph of solubilized AcChR before (A) and after (B) papain treatment. The low magnification micrographs (×80 000) show the presence of doughnut-shaped structures in both preparations. In the higher magnification insets (×140 000) dimers are designated by arrows and monomers by arrowheads. Although the fundamental appearance of normal (A) and proteolyzed (B) AcChR is similar, the papain-treated molecules tend to clump and are contrasted somewhat more diffusely by the negative stain.

only of the α subunit (Sobel et al., 1977; Merlie et al., 1977; Shorr et al., 1978). Demonstration that these fragments remain associated with the macromolecule accounts for the anomaly that a 250 000 molecular weight AcChR reported to be composed entirely of 38 000 molecular weight α subunits in fact had only about one-fourth the [^{125}I]-α-BGT binding capacity expected. Demonstration that the fragments of β, γ, and δ remain associated with proteolyzed AcChR is also important for interpreting the results of Bartfield & Fuchs (1979). They trypsinized AcChR to the point at which a 27 000 molecular weight fragment of α is prominent on electrophoresis. These authors assume that their preparation consists entirely of this peptide, but this, in fact, is very unlikely to be true.

The relative resistance of α to proteolysis by papain and some other proteases is surprising because its amino acid composition does not differ remarkably from that of β, γ, or δ (Lindstrom et al., 1979b; Vandlen et al., 1979). Proteolysis of ^{125}I-labeled AcChR followed by dissociation in $NaDodSO_4$ and immunoprecipitation with antisubunit antibodies demonstrates that the resistant α band is recognized only by antisera to α and not by antisera to any of the other subunits (data not shown). Thus, resistance of α to proteolysis is real rather than a result of accumulation of fragments of β, γ, and δ of the apparent molecular weight of α. The resistance of α to proteolysis does not result from protection of α in the native molecule by the other subunits, because by using individual purified subunits the resistance of α to proteolysis is also apparent (W. Gullick and J. Lindstrom, unpublished results). In any event, the relative resistance of α to proteolysis is not responsible for the remarkable resistance of [^{125}I]-α-BGT binding or carbamylcholine-induced $^{22}Na^+$ influx to proteolysis, because even after treatment of AcChR with the relatively high

amounts of papain required to eliminate all intact α, these functions remain essentially unaltered.

Our results demonstrate that the proteolytic fragments of AcChR remain not only structurally associated but also functionally associated. [^{125}I]-α-BGT binding and to a large extent $^{22}Na^+$ flux remain intact after proteolysis by papain. Pronase and several other proteolytic enzymes also have similar effects (Huganir & Racker, 1980). That AcChR retains functional activity after proteolysis should not be surprising because muscle is routinely treated with collagenase and subtilisin to digest the basement membrane and permit removal of the nerve ending prior to electrophysiological studies of AcChR function (Betz & Sakmann, 1973).

Assay of carbamylcholine-induced $^{22}Na^+$ influx in reconstituted vesicles is a very sensitive method for assaying AcChR function, because $^{22}Na^+$ flux is directly proportional to the number of active AcChRs (Lindstrom et al., 1980a). This contrasts with native vesicles, in which $^{22}Na^+$ flux is limited by equilibration of the external $^{22}Na^+$ with the internal volume of the vesicles due to the high concentration of AcChR (Moore et al., 1979; Lindstrom et al., 1980b). Demonstration that the AcChR cation channel remains active after proteolysis explains the results of Huganir et al. (1979), who purified and reconstituted AcChR from *T. californica* and reported that it contained no γ subunit and a doublet at the α position. These observations are the result of proteolysis due to the omission of EDTA and iodoacetamide during purification, and these authors now observe α, β, γ, and δ subunits in their purified AcChR (Huganir & Racker, 1980).

The resistance of proteolytically nicked fragments of AcChR to disaggregation demonstrates that the structure of this macromolecule depends on the intimate noncovalent association of its parts. The resistance of proteolytically nicked AcChR to loss of ligand binding or cation channel function indicates that the function of this macromolecule depends on noncovalent interactions between its components. The resistance of AcChR to disaggregation into subunits by 4 M urea (Lindstrom et al., 1976) or extensive proteolysis suggests that the intimate association of its subunits is very important and that it may be difficult or impossible to dissociate its subunits and then reconstitute them and regain function.

Added in Proof

Klymkowsky et al. (1980) recently observed that AcChRs in vesicles of native membrane were more sensitive to proteolysis after alkaline extraction. All subunits could be degraded. This was accompanied by clumping of the proteolyzed AcChR in the plane of the membrane and blebing of "minivesicles". Note the similarity between this clumping of proteolyzed AcChR in membranes and the clumping of solubilized proteolyzed AcChR which we observed (Figure 2B).

Saitoh et al. (1980) recently reported that a Catt-dependent protease is responsible for degrading β, γ, and δ subunits of AcChR in membranes prepared by the method of Sobel et al. (1977) and confirmed previous observations that this protease could be effectively inhibited by chelating agents (Damle & Karlin, 1978; Vandlen et al., 1979; Schiebler & Hucho, 1978). Thus, there is now general agreement that *Torpedo* AcChR is composed of α, β, γ, and δ subunits in the mole ratio $\alpha_2\beta\gamma\delta$ (Damle & Karlin, 1978; Lindstrom et al., 1979b; Raftery et al., 1980). This is consistent with our observation that these subunits remain both associated and functional despite reconstitution and proteolysis.

Acknowledgments

We thank Vernita Hudson, John Cooper, Brett Einarson, and Thomas Deerinck for technical assistance and Robert Anholt for helpful discussions.

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Proton Nuclear Magnetic Resonance Study of the Self-Complementary Hexanucleotide $d(pTpA)_3$ and Its Interaction with Daunomycin†

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ABSTRACT: The helix–coil transition of the self-complementary hexanucleotide $d(pTpA)_3$ has been studied in 1 M NaCl by high-resolution proton nuclear magnetic resonance spectroscopy. Almost all of the 12 resonances deriving from the three environments of the four nucleotide protons have been assigned to the central, internal, or terminal nucleotides. At 5 °C, the effect of extensive fraying is evident since the central base pairs exhibit only 20% of the chemical shifts observed for poly(dA-dT)·poly(dA-dT) accompanying denaturation. Daunomycin interacts with the hexanucleotide duplex at 5 °C and stabilizes it by 21 °C at a drug/nucleotide ratio of 0.063 (i.e., drug/hexanucleotide duplex ratio of 0.75). The chemical shifts of the drug protons suggest that ring D of daunomycin does not overlap significantly with the central base pairs of the hexanucleotide and that it extends out from the "helix". This information, together with studies of space-filling models of the complex, suggests that rings B and C of daunomycin overlap with adjacent base pairs and are skewed with respect to the base pairs.

Daunomycin and its analogue, adriamycin, are both used extensively for the treatment of a variety of forms of cancer (Di Marco et al., 1975) with an increasing emphasis on their clinical use in combination therapy (Keiser & Capizzi, 1977). The chemical, biochemical, and physicochemical aspects of these drugs have been extensively reviewed by Arcamone (1978). Although several modes of action of these drugs have been proposed and have been reviewed by Neidle (1978), the dominant mode of action is still thought to be due to their ability to intercalate into DNA, resulting in the inhibition of both DNA polymerase and RNA polymerase (Neidle, 1978). Unfortunately, the use of these drugs for cancer chemotherapy remains limited by their associated cardiotoxicity (Lefrak et al., 1973; Gilladoga et al., 1976) and adverse side effects common to all cytotoxic drugs (Di Marco et al., 1975). Several attempts have been made to alleviate the cardiotoxicity and other side effects by encapsulating the drugs in various ways (Gregoriadis, 1977) and by administering a drug–macromolecule complex (Cornu et al., 1974). An alternative approach has been to modify the existing drugs in an attempt to produce derivatives with decreased side effects. However, this approach requires a detailed knowledge of the nature of the receptor site. Although the DNA intercalation mechanism is acknowledged as the dominant mode of action, there has been a surprising lack of detailed information about the exact geometry of the DNA–daunomycin complex. Pigram et al. (1972) have used X-ray diffraction to obtain information about the complex in the solid state. Recently, proton NMR studies by Patel & Canuel (1978) and Patel (1979) have provided insight into the geometry of the complex in solution by using dG-dC-dG-dC and poly(dA-dT) as model DNA compounds.

We have attempted to obtain details of the geometry of the complex formed between daunomycin and the self-complementary hexanucleotide $d(pTpA)_3$ by using high-resolution proton NMR spectroscopy. This hexanucleotide was selected as a model DNA compound, suitable for NMR studies, on the basis of a compromise between the desire to have a low molecular weight duplex (with a short correlation time) and the requirement that the duplex be stable above 0 °C. Solubility problems precluded the use of the comparable G-C containing hexanucleotide, $d(pGpC)_3$. Since the hexanucleotide $d(pTpA)_3$ has not previously been studied by high-resolution proton NMR, it was first necessary to assign all of the resonances of the hexanucleotide, since each nucleotide exists in three

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0006-2960/80/0419-4795$01.00/0