1)O-SUBSTITUTED SERINE AND THREONINE RESIDUES IN ACETYLCHOLINE RECEPTOR PROTEIN FROM *TORPEDO CALIFORNICA*

2)SYNTHESIS OF A NEW FLUORESCENT PROBE FOR LIGAND BINDING TO ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA*

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ABSTRACT

Part I deals with chemical studies performed on purified acetylcholine receptor from Torpedo Californica designed to document the presence and nature of O-substituted serine and threonine residues. Treatment of receptor with methylamine in base and analysis of the products formed showed 24 O-substituted serine residues per 300,000 daltons protein and the presence of O-substituted serine in all four subunits. The amount of O-substituted threonine present in receptor was estimated by treatment with alkaline sodium borohydride, suggesting 25 such residues per 300,000 daltons protein. Evidence is presented suggesting that at least half of this O-substitution to serine and threonine is accounted for by O-glycosyl linkage to carbohydrate chains of short length.

Part II deals with synthesis of a fluorescent probe for ligand binding to acetylcholine receptor. The probe synthesized contains a highly fluorescent moiety and a photo-induced reactive azide group, and is a suicide probe in that it contains an acetylcholine moiety which should direct it to the ligand binding site. This latter moiety can be removed after reacting the azide to leave a fluorescent probe located adjacent to the ligand binding site.

TABLE OF CONTENTS

PART 1

O-SUBSTITUTED SERINE AND THREONINE RESIDUES IN ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA*

INTRODUCTION

At the neuromuscular junction, as well as at many synapses, neurotransmission occurs by the secretion of acetylcholine(AcCh)[1] from the presynaptic nerve, diffusion of AcCh across the synaptic cleft and interaction of AcCh with the membrane bound acetylcholine receptor(AcChR) protein, causing permeability changes to certain ions and generation of a postsynaptic potential. Much of the physiological and pharmacological study on the AcChR has been done on the electroplaque of the electric eel, Electrophorus electricus(Cohen and Changeux, 1975; Rang, 1975) as well as on vertebrate neuromuscular junction. Biochemical studies have largely been performed on AcChR isolated from the electric organs of electric fish and eels, such as Narcine entemedor (Schmidt and Raftery, 1972); Torpedo californica(Schmidt and Raftery, 1973; Weill et al., 1974); Torpedo marmorata(Karlsson et al., 1972; Cohen et al., 1972;

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[1]Abbreviations:AcCh, acetylcholine; AcChR, acetylcholine receptor; AMAP, α-amino-β-methylaminopropionic acid; BSA, bovine serum albumin; BSM, bovine submaxillary gland mucin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate.

Eldefrawi and Eldefrawi, 1973; Gordon et al., 1974); Electrohporus electricus(Olsen et al., 1972; Biesecker, 1973; Karlin and Cowburn, 1973; Klett et al., 1973; Chang, 1974); and Torpedo nobiliana(Ong and Brady, 1974).

There remains considerable controversy considering the most basic structural aspects of the AcChR, despite years of work with highly purified material. The molecular weight of purified Torpedo AcChR has been estimated using several methods, resulting in a wide range of values from 190,000(Gibson et al., 1976) to 500,000 (Raftery, et al., 1972). Several researchers have found the presence of two molecular forms of purified AcChR in nonionic detergent solution(Vandlen and Raftery, 1978; Gibson et al., 1976). In sucrose density gradient, protein is observed at 13S and 9.5S, the former proposed to be a dimer of the latter. Vandlen and Raftery(1978), using PAGE, estimated the molecular weight of the dimer to be 700,000 and the monomer 385,000. The dimer can be converted into the monomer by the addition of a reducing agent(Witzemann and Raftery, 1977; Chang and Bock, 1977; Hamilton et al., 1977), suggesting presence of a disulfide linkage to produce the dimer. Binding of radiolabeled snake toxin specific to AcChR showed four binding sites per 360,000 daltons of protein(Vandlen and Raftery, 1978).

When subjected to denaturing SDS PAGE, protein bands from purified AcChR of molecular weight 65,000, 60,000, 50,000 and 40,000 are often observed(Vandlen and Raftery, 1978; McNamee et al., 1975; Weill et al., 1974). The stoichiometry of these bands varies considerably and their relevance has recently been brought into question by Sobel et al(1977), suggesting some may be artifactual in nature. All four subunits are glycoproteins and have very similar amino acid compositions(Vandlen and Raftery, 1978).

Considerable evidence has been reported demonstrating phosphorylation of specific membrane proteins at various types of synapses. Greengard(1976) has shown that the postsynaptic actions of some neurotransmitters might be mediated by phosphorylation of specific membrane proteins in the postsynaptic cells. The most extensively studied preparation has been the mammalian superior cervical sympathetic ganglion, where a cyclic Adenosine monophosphate-dependent phosphorylation process occurs and is coupled to the activation of a dopamine receptor, leading to the generation of the slow inhibitory postsynaptic potential. In general, phosphorylated proteins have been shown to incorporate the phosphate in ester linkage to serine or threonine residues, with only a few cases documented of phosphate linked through an amide to arginine.

Work on the composition of Torpedo cholinergic synaptic vesicles has shown that in addition to AcCh, they contain Adenosine Triphosphate(Whittaker et al., 1972; Dowdall et al., 1974). Teichberg and Changeux (1976) found a difference in isoelectric points between the junctional and extrajunctional AcChR of Electrophorus electricus and suggested this observation could be explained by bound phosphate in the junctional AcChR. The two observations combined suggest some link between phosphorylation of AcChR and its role in synaptic transmission or formation.

Recently there have been reports showing evidence of an in vitro phosphorylation of nicotinic AcChR from the electric organs of Torpedo californica(Gordon et al., 1977) and Electrophorus electricus(Teichberg et al., 1977). However, it is not known whether such phosphorylation of the receptor actually occurs in vivo or was merely the artifact of the in vitro system. This work concerns the study by chemical modification of O-substituted serine and threonine residues in AcChR with the aim of determining the number of such residues in vivo and the substituents to which they are attached.

EXPERIMENTAL

MATERIALS

Torpedo californica were obtained locally in Pacific waters. Bovine Serum Albumin(Cohn Fraction V), Bovine Submaxillary Gland Mucin(Type 1), D,L, α-amino-n-butyric acid were obtained from Sigma Chemical Company; Phosphatidylserine from Supelco, Inc.; Potato Acid Phosphatase(A Grade, Lot 701120, 65 I.U./ml @25° C) from Calbiochem; Reagents for gel electrophoresis from BioRad Labs; Sodium dodecyl sulfate from Pierce Chemical Company. All gel electrophoresis molecular weight standards were obtained from Sigma Chemical Company except catalase from Boehringer-Mannheim Company. α-amino-β-methylaminopropionic acid was synthesized from α-acetamidoacrylic acid according to Vega et al., 1968. The compound was recrystallized from aqueous ethanol to give a white powder of m.p. 165-7° C(uncorrected), and the structure was confirmed by ^{1}H-NMR. All other chemicals were of the highest purity commercially available.

β-Casein was purified according to the method of Manson et al., 1971. This yielded 1.67 gm of purified material from 1 liter of raw milk and was stored as a freeze-dried powder at 0° C.

METHODS

AcChR was purified by affinity chromatography (Schmidt and Raftery, 1972) from the electric organs of

Torpedo californica as previously described(Schmidt and Raftery, 1973; Vandlen et al., 1976).

SDS PAGE was performed essentially as described by Fairbanks et al., 1971 in buffers containing 0.1% SDS. The samples were denatured in 2 to 5% SDS with 5% β-mercaptoethanol or 10^{-4} M DTT for 1.5 minutes at 100° C. The gels were stained with 0.05% Coomassie Brilliant Blue and destained.

Preparative scale SDS PAGE as described by Claudio and Raftery,(1977)was utilized for isolation of AcChR subunits. The standard proteins used and their subunit molecular weights were:Myosin(210,000)Phosphorylase A (93,000)BSA(68,000)Catalase(60,000)Rabbit muscle aldolase(40,000) and Myoglobin(17,000).

Quantitation of neutral sugars was achieved by ion exchange chromatography in 75% ethanol(Heller and Raftery, 1976). Amino sugars were determined during amino acid analysis following hydrolysis in 6N HCL for 20 hours.

For amino acid analysis, protein samples were dialyzed for 24 hours to remove salts and excess detergent before hydrolysis in vacuo with 6N HCL at 110° C for 20 hours. Hydrolysates were dried under vacuum and analyzed according to Spackman et al., 1958 modified using a basic buffer containing 1 M NaCl, 0.1 M NaCitrate, 0.1% Brij 35, pH 8.9 on a Beckman model 120B Amino Acid Analyzer.

Proteins were oxidized by the method of Hirs(1956). Reduction and carbamidomethylation was carried out in .5 M Tris-HCl, pH 7.8, containing 1% Triton X-100 in the case of AcChR samples. These solutions(2mg/ml in protein) were brought to 0.1 M in β-Mercaptoethanol and incubated at room temperature for 3 hours. Solid Iodoacetamide was then added to a final concentration of 0.2 M and the solution stirred in the dark at room temperature for 1/2 hours. Samples were extensively dialyzed against several changes of water at 4° C and lyophilyzed.

The procedure for methylamine reaction was adapted from that described by Kolesnikova et al.(1974). Protein samples(1-5 mg) were taken up in 1 ml 1 N NaOH, 1 M CH_3NH_2. The solutions were stirred at 37°C for 8 hours, then neutralized(to Litmus) by addition of 6 N HCl. Samples were extensively dialyzed against several changes of water at 4°C before hydrolysis.

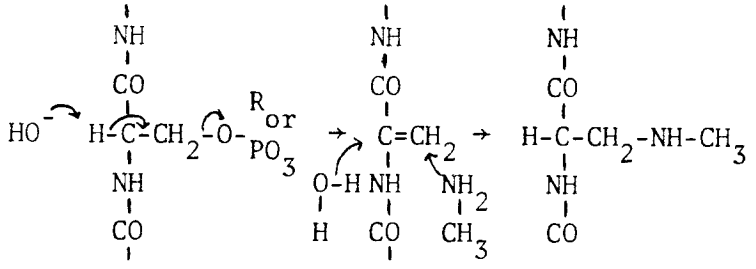
Reaction of proteins to Na_2SO_3 was essentially by the method of Weber and Winzler(1970). Proteins were taken up in .25 N NaOH, .5 M Na_2SO_3(freshly prepared) to a concentration of 5 mg/ml. The solutions were incubated at room temperature for 70 hours, neutralized (to Litmus) by addition of 6 N HCl and dialyzed against water at 4° C before hydrolysis.

The procedure for reaction in base to sodium borohydride was as described by Heller and Raftery(1976).

RESULTS

METHYLAMINE REACTION

The methylamine reaction was used to determine quantitatively the amount of O-substituted serine residues in AcChR. The reaction involves α-elimination in base to produce a dehydroalanine intermediate followed by an α,β-nucleophilic addition with CH_3NH_2 as the nucleophile, as shown:



The product formed, α-amino-β-methylaminopropionic acid(AMAP), is stable to acid hydrolysis and can be separated on the Amino Acid Analyzer as shown in Figure 1. The AMAP produced in reaction to protein was determined quantitatively by comparison with a chemically synthesized standard.

Since cysteine can react under the experimental conditions to give the same product as does O-substituted serine, proteins were either oxidized or reduced and carbamidomethylated before reaction. The results of this reaction with standards is given in Table 1. By oxidation the reaction was minimized to yield an amount of AMAP equivalent to only 8% of the cysteine originally present in BSA. Quantitative reaction was shown with

FIGURE 1

Separation of α-amino-β-methylaminopropionic acid. Separation was performed on a 25 × 1 column of Dowex-50 resin eluted with .35 M NaCitrate, pH 5.3 at 68 ml/hr at 60°C. Sample contained 125 nmole of each amino acid and 100 nmole AMAP.

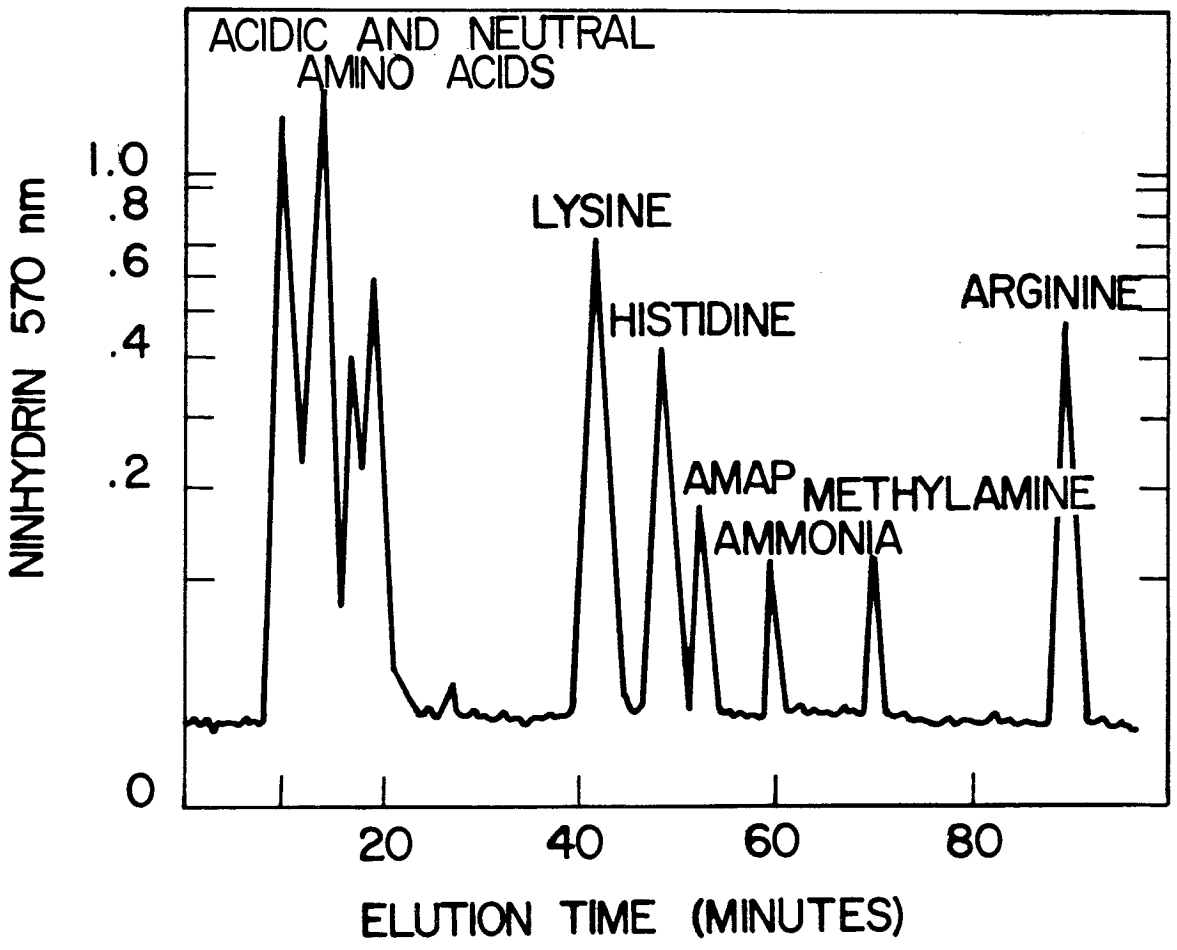

ACIDIC AND NEUTRAL
AMINO ACIDS
LYSINE
HISTIDINE
AMAP
AMMONIA
METHYLAMINE
ARGININE
NINHYDRIN 570 nm
1.0
.8
.6
.4
.2
0
20
40
60
80
ELUTION TIME (MINUTES)

TABLE 1

METHYLAMINE REACTION-STANDARDS

PROTEIN	O-SUBSTITUTED SERINE	CYSTEINE	AMAP	N
ox. BSA		6.30[1]	.48±.03	2
β-Casein	Phosphoserine(2.4)[2]	0	2.4±.03	4
ox. BSM	Glycosidylser(2.8)[3]	1.4[4]	0.8	1
r.c. BSM	Glycosidylser(2.8)[3]	1.4[4]	1.2±.01	2

Values in mole % with standard deviations. ox. = oxidized; r.c. = reduced and carbamidomethylated. N = number of experiments. 1 = Cysteine in hydrolysis of unmodified protein; 2 = Ribadeau-Dumas et al., 1972; 3 = Determined by sulfite reaction on r.c. material, calculated from dry weight; 4 = Cysteine in hydrolysis of unmodified protein, calculated from dry weight.

phosphoserine residues in β-Casein and less quantitative with glycosylated serine residues in BSM.

Reaction of methylamine in base with purified AcChR and its subunits is summarized in Table 2. The AcChR and all its subunits, with the possible exception of the 50,000 dalton polypeptide, were found to yield AMAP as product under these conditions.

Since phosphatidylserine is prevalent in Torpedo californica membranes(Michaelson and Raftery, 1974), the reaction could conceivably have occurred with some tightly bound phosphatidylserine which had been co-purified with the AcChR. However, reaction with free phosphatidylserine(1000 nmole) showed no AMAP(detection limit ≃ 1 nmole).

SULFITE REACTION

In order to determine the extent of O-substitution to threonine in AcChR, reduced and carbamidomethylated samples were reacted in base with Na_2SO_3. This reaction gives cysteic acid from O-substituted serine residues and 2-amino-3-sulfonyl butyric acid from O-substituted threonine residues, which comigrate when analyzed at both pH 3.2 and pH 1.8(Simpson et al., 1972). Results of one such experiment are summarized in Table 3. As can be seen the decrease in serine and threonine content in both BSM and AcChR roughly corresponds to the amount of cysteic acid and 2-amino-3-sulfonylbutyric

TABLE 2

METHYLAMINE REACTION-AcChR

AcChR PREP	CYSTEINE[1]	AMAP	# AMAP/ MOLECULE	N
ox. whole	1.3	.98±.20	-	8
r.c. whole	1.3	1.1±.11	-	2
ox. 65K	1.0	.67±.18	4.0±1.1	2
ox. 60K	1.2	.72±.36	3.6±1.8	3
ox. 50K	0.8	.22±.20	1.1±1.0	2
ox. 40K	0.9	.64±.04	2.1±0.1	2

Values in mole % with standard deviations. ox. = oxidized, r.c. = reduced and carbamidomethylated, N = number of experiments. 1 = Cysteine present in unmodified protein.

TABLE 3

SULFITE REACTION-BSM AcChR

PROTEIN	THR	SER	DECREASE IN SER THR	CYSTEIC ACID 2-AMINO-3-SULFONYLBUTYRIC ACID
BSM	7.7	8.2	-	-
$+SO_3$	4.6	5.4	5.7	6.3
AcChR	6.2	6.3	-	-
$+SO_3$	5.4	4.9	3.2	2.3

Values in mole %. All samples were reduced and carbamidomethylated prior to reaction. Values for BSM calculated from dry weight. The color value of 2-amino-3-sulfonylbutyric acid was assumed to be the same as that of Cysteic acid. Thr = threonine, Ser = serine.

acid produced.

AcChR samples that had been reacted in base with Na_2SO_3 were analyzed for neutral sugars in order to determine if O-glycosidic bonds were being cleaved. Results are given in Table 4.

SODIUM BOROHYDRIDE REACTION

A direct measurement of the number of O-substituted threonine residues in AcChR was made by reaction to sodium borohydride in base. This reaction consists of α-elimination in base to produce a dehydrobutyric acid residue in the protein which is then reduced to give α-amino-n-butyric acid. The corresponding reaction with serine produces alanine. The α-amino-n-butyric acid was separated and determined quantitatively by comparison to standard α-amino-n-butyric acid. This reaction with AcChR yielded 1.0±.15 mole % α-amino-n-butyric acid(3 experiments).

TABLE 4

SULFITE REACTION:

EFFECT ON CARBOHYDRATE CONTENT OF AcChR

PROTEIN	MANNOSE	GLUCOSE	GLUCOSAMINE	GALACTOSE	TOTAL SUGAR LOSS
AcChR	1.70	0.15	1.25	0.27	-
$+SO_3$	1.18	0.12	0.76	0.17	1.14
$+SO_3$	1.27	0.13	0.73	0.18	1.06

Values given in residues carbohydrate per 100 amino acid residues.

DISCUSSION

The total amount of O-substitution to serine and threonine residues in AcChR has been determined. It should be noted that reaction in base with methylamine occurs not only with phosphoserine, but with glycosylated serine residues, the latter not having been previously reported. The data by reaction to methylamine and that to sulfite suggest approximately .98 mole % substituted serine in AcChR and its presence in all subunits. Thus, 15 % of the serine present in AcChR contains O-substitution. In addition to these residues, sulfite reaction to AcChR suggests there is approximately .74 mole % O-substituted threonine. The direct estimate of O-substituted threonine content by reduction in base gives a somewhat higher value of 1.0 mole %, corresponding to 16% of the total threonine present.

Concerning the nature of this substitution, Wilson Wu of this laboratory has found that AcChR contains .28 mole % phosphoserine and no phosphothreonine within the detection limits of the technique(unpublished results). The observation here that under conditions employed for modification of O-substituted residues, 33 % of the neutral sugars present in AcChR are cleaved suggests that another portion of the substitution is of glycosidic nature.

Assuming a molecular weight for AcChR of 300,000 daltons, the number of residues of O-substituted serine and threonine, phosphoserine and base-labile sugar is given in Table 5. The number reported by Wilson Wu for phosphoserine presence in AcChR is supported by the observation that treatment of AcChR with potato acid phosphatase, which removed 75% of the phosphoserine content of β-Casein, reduced the number of AMAP observed without treatment by 6 residues/300,000 daltons protein. If it is then assumed that the number of phosphoserine residues is a good estimate, in order to account for the numbers observed many of the serine and threonine residues joined to carbohydrate must be joined to single sugars, rather than extended chains. The functional significance of this observation cannot be concluded at this time.

TABLE 5

O-SUBSTITUTION OF SERINE

AND THREONINE IN AcChR

O-SUBSTITUTED SERINE	O-SUBSTITUTED THREONINE	O-SUBSTITUTED SER + THR	P-SER	BASE LABILE SUGAR
24[1]	25[2]	42[3]	7[4]	26[3]

Values given in residues/molecule assuming a molecular weight for AcChR of 300,000. 1 = Determined by methylamine reaction, 2 = Determined by sodium borohydride reaction, 3 = Determined by sulfite reaction, 4 = Wilson Wu, unpublished results. Ser = Serine, Thr = Threonine, P-Ser = Phosphoserine.

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PART 2

SYNTHESIS OF A NEW FLUORESCENT PROBE FOR LIGAND BINDING TO ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA*

INTRODUCTION

The AcChR[1] has been studied with respect to cholinergic ligand binding by several methods. These include equilibrium dialysis or centrifugation studies of radioactive ligand binding(Eldefrawi et al., 1971; Moody et al., 1973; Chang, 1974; Meunier et al., 1974; Raftery et al., 1974), inhibition of the initial rate of formation of AcChR-radioactive neurotoxin complex by cholinergic ligands(Weber and Changeux, 1974; Moody et al., 1973; Schmidt and Raftery, 1974; Fulpius et al., 1974; Meunier et al, 1974) and use of paramagnetic and fluorescent probes. The latter probes have been used specifically to study conformational changes in AcChR caused by ligand binding. Wieland et al.,1976, used the paramagnetic resonance of a spin labeled-ligand for such a purpose, while others have used intrinsic fluorescence(Bonner et al., 1976; Barrantes, 1976) or

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[1]Abbreviations:AcChR, acetylcholine receptor; ADAT, α-acetylcholine 2,-(5-dimethyl-aminonaphthalene-1-sulfonamide)-3,-(p-azidophenyl)thiolpropionate; Chol, propane-1-trimethylammonium iodide; DAP, bis(3-aminopyridinium)1,10-decane diiodide; DCC, dicyclohexylcarbodiimide; DNS,(5-dimethyl-aminonaphthalene-1-sulfonyl-); DTT, dithiothreitol; Phe, phenylalanine

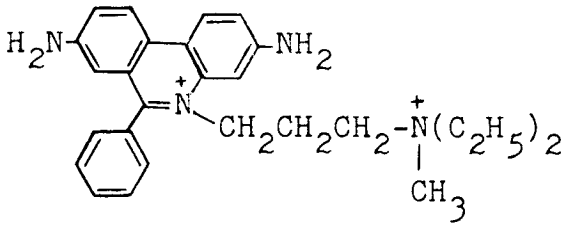
extrinsic fluorescent probes such as DAP(Martinez-Carrion and Raftery, 1973; Bode et al., 1976), DNS-Chol (Cohen and Changeux, 1973), quinacrine(Grunhagen and Changeux, 1975; Changeux et al., 1976), propidium(Sator et al., 1977) and ethidium(Schimerlik and Raftery, 1976). The structures of these compounds are given in Figure 1.

This work deals with the synthesis of a new extrinsic fluorescent probe for ligand binding studies on AcChR. This probe, ADAT(Structure given in Figure 1), contains three features desirable for such studies, which are a fluorophor(DNS), a reactive group for binding to the protein(Azide) and a removable ligand binding site-director(Acetylcholine). Such a probe would be directed with high specificity to the cholinergic ligand binding site of AcChR as suggested by the nicotinic activity of thioglycolcholine(Janczarski et al., 1968), could then be anchored to the protein via photolytic reaction of the azide and the acetylcholineSH residue removed by mild base hydrolysis of the labile thiolester. This would leave a fluorophor bound specifically adjacent to the ligand binding site which should be an excellent reporter of ligand binding through changes in its immediate environment and quantum yield of fluorescence.

FIGURE 1

Structures of fluorescent probes
for ligand binding to AcChR

Propidium



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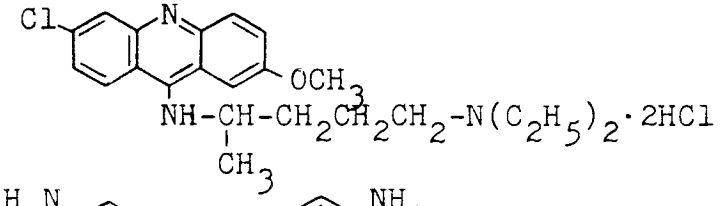

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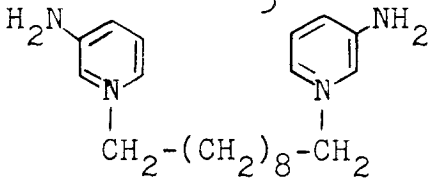

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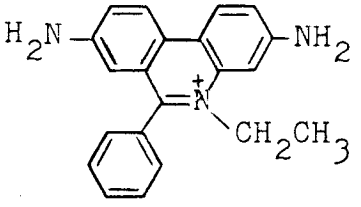

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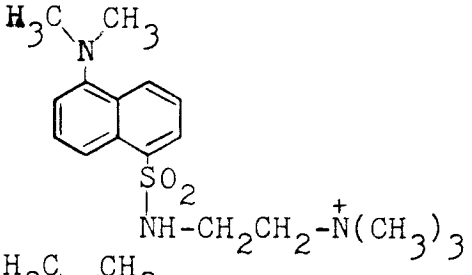
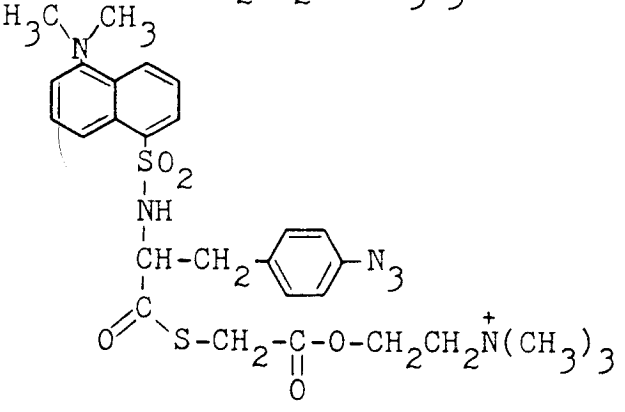


ADAT



EXPERIMENTAL

MATERIALS

DNS-Cl and dithiodiglycollic acid were obtained from Aldrich Chemical Co.; pNO_2-Phe from Bachem, Inc.; dithiothreitol from Sigma Chemical Co.; N-dimethylaminoethanol from Eastman Organics; dicyclohexylcarbodiimide and methyl iodide from Matheson Coleman and Bell Manufacturing Chemists; pre-coated TLC plastic sheets of silica gel 60 from EM Laboratories, Inc..

METHODS

TLC was performed on silica gel plates and sprayed for tertiary amines(Lane, 1965), esters(Whittaker and Wilesundera, 1952) or disulfides(Toennies and Kolb, 1951). Fluorescent spots were visualized using the long wave setting of a UV lamp.

^{1}H-NMR was performed on a Varian EM-390; UV-Vis spectra on a Cary 118; IR spectra on a Beckman IR 4240; fluorescent spectra on a Perkin Elmer MPF-4.

DNS-pNO_2Phe[2]

To a solution of 1.90 gm(9.0 mmol) pNO_2Phe and 31.2 gm(376 mmol) $NaHCO_3$ in 740 ml H_2O was added 370 ml

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[2]All products exhibited correct ^{1}H-NMR and IR spectra and ran as single spots in TLC in three different solvent systems.

acetone containing 4.80 gm(18 mmol) DNS-Cl. The solution was stirred in the dark at room temperature for 24 hours. The pH was then adjusted to 5 with conc. HCl and the acetone removed under reduced pressure. This aqueous solution, the pH of which had risen to 8, was extracted with 3 150 ml portions of ether and the resultant clear orange solution brought to pH 3, at which point a light yellow precipitate appeared. This was filtered and dried to yield 3.06 gm DNS-pNO_2Phe(66% yield), m.p.=215-216° C.[3] The product exhibited absorbance maxima at 334, 256 and 216 nm and maximum fluorescence with excitation at 329 nm and emission at 405 nm. Found in material dried in vacuo over P_2O_5 24 hours: C=54.3%, H=4.75%, N=9.54%. $C_{21}H_{20}N_3SO_6$ requires C=57.0%, H=4.52%, N=9.50%.

DNS-pNH_2Phe

2.40 gm(5.4 mmol) DNS-pNO_2Phe in 260 ml ethanol containing 75 mg PtO_2 was stirred in the dark at room temperature in a hydrogen atmosphere kept constant at 1 atmosphere pressure for 22.5 hours(total H_2 uptake= 437 ml=19.4 mmol). The solution was filtered and dried to yield 1.68 gm DNS-pNH_2Phe(75% yield), m.p.=129-131° C. The product exhibited absorbance maxima at 330, 304, 247 and 218 nm and maximum fluorescence with excitation

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[3]All melting points uncorrected.

at 345 nm and emission at 507 nm. Found in material dried in vacuo over P_2O_5 24 hours: C=59.5%, H=5.88%, N=9.55%. $C_{21}H_{22}N_3SO_4$ requires C=61.2%, H=5.34%, N=10.2%.

DNS-pN_3Phe

To 1.60 gm(3.9 mmol) DNS-pNH_2Phe in 80 ml H_2O in the dark in an ice bath was added 125 2° C H_2O containing 1.30 ml conc. HCl(15.5 mmol). The solution was stirred in the dark in an ice bath and 125 ml 2°C H_2O containing 1.08 gm(15.5 mmol) $NaNO_2$ was added over 30 minutes, followed by the addition of 125 ml 2°C H_2O containing 1.01 gm(15.5 mmol) NaN_3 also over 30 minutes. The solution was allowed to warm slowly to room temperature and the pH was then raised from 5.5 to 10 by the addition of saturated NaOH. The red solution was filtered and brought to pH 3 by the addition of conc. HCl at which point a yellow precipitate formed. The precipitate was filtered and dried, taken up in 20 ml 30°C acetone, diluted with 50 ml 30° C H_2O, cooled slowly to 0°C and kept at that temperature for 2 days. The resultant reddish crystals were filtered and dried to yield .90 gm DNS-pN_3Phe(53% yield), m.p.=128-129.5°C. The product exhibited absorbance maxima at 334, 290, 252 and 212 nm with decreased absorbance at 250 nm when exposed to UV irradiation. Maximum fluorescence was attained with excitation at 350 nm and emission at

514 nm. Found in material dried in vacuo over P_2O_5 24 hours: C=55.7%, H=4.92%, N=15.2%. $C_{21}H_{20}N_5SO_4$ requires C=57.5%, H=4.57%, N=16.0%.

Dithiodiglycollyl Choline Diiodide

Dithiodiglycollyl choline diiodide was synthesized from dithiodiglycollic acid, N-dimethylaminoethanol and methyl iodide via the diacid chloride according to Gulland et al., 1940. The product obtained had a melting point of 152-154° C.

ADAT

ADAT was synthesized from dithiodiglycollyl choline diiodide and DNS-pN_3Phe by reduction of the disulfide to the free mercaptan and coupling it with DNS-pN_3Phe using DCC as condensing agent(Grunwell and Foerst, 1976) as follows: 200mg(0.40 mmol) dithiodiglycollyl choline diiodide and 62.0 mg(0.40 mmol) DTT in 25 ml dry methanol were stirred at room temperature 1 hour, and then added to 10 ml dry acetone containing 176 mg(0,40 mmol) DNS-pN_3Phe and 83 mg(0.40 mmol)DCC which had previously been stirred in the dark at room temperature for ½ hour. The resulting solution was stirred in the dark at room temperature for 3 hours followed by removal of sovent under reduced pressure. The residue was then taken up in 25 ml acetone and filtered to yield 70 mg of a white powder(m.p. 225-226° C) assumed to be dicyclohexylurea. The solution was dried under reduced pressure and taken

up in 5 ml 30° C ethanol. 1 ml 30° C H_2O was added and the solution cooled slowly to 0°C and kept at that temperature for 2 days. The light yellow precipitate formed was dried to yield 53 mg ADAT(20% yield), m.p.= 188-190° C. The product exhibited absorbance maxima at 282, 274 and 222 nm, all of which were reduced by UV irradiation. Maximum fluorescence was attained with excitation at 383 nm and emission at 520 nm. Found in material dried in vacuo over P_2O_5 24 hours: C=45.41%, H=4.74%, N=10.73%. $C_{27}H_{35}N_6S_2O_5I$ requires C=45.43%, H=4.94%, N=11.77%.

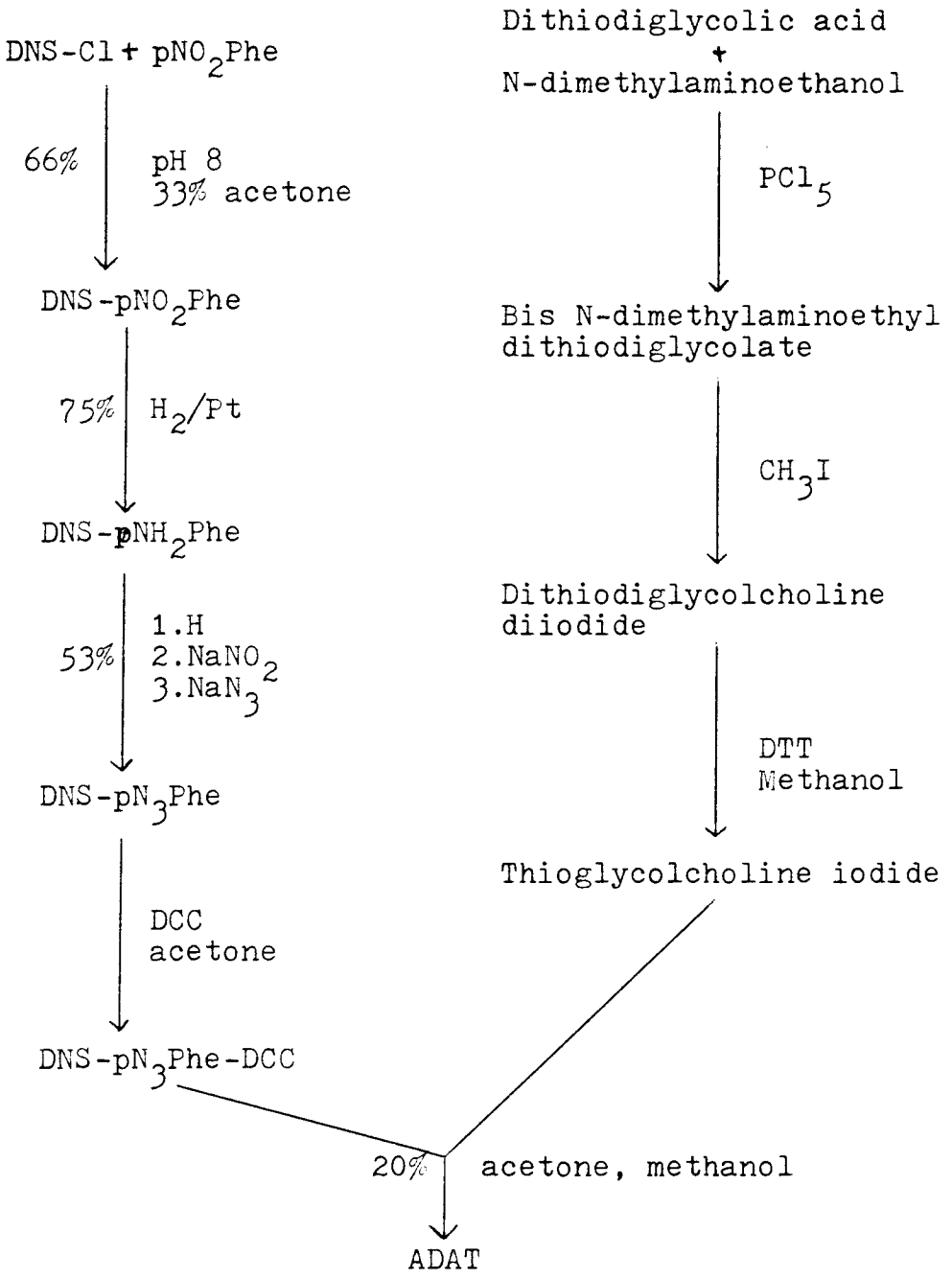
RESULTS AND DISCUSSION

This work describes the successful synthesis of ADAT, a new fluorescent probe for ligand binding to AcChR. The synthetic route, summarized in Figure 2, was devised within the constraint of two chemically labile groups(aromatic azide and thiolester) and with ease of purification in mind.

The synthetic route from DNS-Cl and pNO_2Phe to DNS-pN_3Phe was straightforward and a final product in crystalline form was easily obtained. However, it was desirable to form the thiolester of DNS-pN_3Phe and N-dimethylaminoethyl thioglycolate with DCC as the condensing agent in ether or a similar organic solvent. In this manner, the dicyclohexylurea formed would be insoluble and could be removed by filtration. In addition, quarternization of the amine by addition of methyl iodide would bring the ADAT out of solution as well as affording a simple method for the introduction of radiolable into the compound. The Bis N-dimethylaminoethyl dithiodiglycolate formed in the reaction scheme of Gulland et al., 1940 was as reported a brown oil, and attempts to purify it failed. It was also synthesized via the N-methylpyridinium salt according to the method of Mukaiyama et al., 1975 using 2-iodopyridinium methiodide as starting material, but again a brown oil was

FIGURE 2

Synthetic route to ADAT


DNS-Cl + pNO2Phe
66%
pH 8
33% acetone
DNS-pNO2Phe
75%
H2/Pt
DNS-pNH2Phe
53%
1.H
2.NaNO2
3.NaN3
DNS-pN3Phe
DCC
acetone
DNS-pN3Phe-DCC
Dithiodiglycolic acid
+
N-dimethylaminoethanol
PCl5
Bis N-dimethylaminoethyl
dithiodiglycolate
CH3I
Dithiodiglycolcholine
diiodide
DTT
Methanol
Thioglycolcholine iodide
20%
acetone, methanol
ADAT

obtained. The result was quarternizing the amine before formation of the thiolester as reported.

Initial experiments using ADAT would test for a change in fluorescence between free and AcChR-bound ADAT. One should then see if this change in fluorescence is reversed by incubation with cholinergic ligands or neurotoxins. Incubation of ADAT with AcChR followed by photolysis of the solution and extensive dialysis before looking for remaining fluorescence would test the ability of the azide to react with the protein. Treatment of the ADAT-AcChR to pH 8 solution followed by dialysis should provide evidence by the sodium cyanide-sodium nitroprusside test for disulfide in the dialysate resulting from cleaved thioglycolcholine which would quickly oxidize to the disulfide in oxygenated solution. Given positive results to the above tests, the DNS-pAcChRPhe complex could be studied for fluorescence changes with addition of ligands, first at equilibrium and then in the stopped-flow. Since the reagent should be highly specific for the ligand binding site of AcChR, studies could be performed with AcChR in pure, solubilized form or in membranes with little resulting background fluorescence.

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SUMMARY OF RESULTS

The results in Part 1 consist of studies by chemical modification on the number of residues of O-substituted threonine and serine in the acetylcholine receptor from Torpedo californica. There were found to be 24 O-substituted serine and 25 O-substituted threonine residues/300,000 daltons proein. Under the modification conditions used, 26 residues sugar/300,000 daltons protein were cleaved, suggesting much of the substitution is to carbohydrate chains of short length.

Part 2 describes the successful synthesis of α-acetylcholine 2,-(5-dimethylaminonaphthalene-1-sulfonamide)-3,-(p-azidophenyl) thiolpropionate. The named compound is to be used as a fluorescent probe for ligand binding to acetylcholine receptor in solubilized form and in membranes.