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Activation of the Frog Sartorius Acetylcholine Receptor by a Covalently Attached Group

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Summary. The frog sartorius motor endplate was treated with the specific disulfide bond reducing agent dithiothreitol and subsequently exposed to a covalently reacting compound (the nitrophenyl ester of *p*-carboxyphenyltrimethylammonium iodide, NPTMB) known to activate the dithiothreitol-reduced acetylcholine receptor in *Electrophorus* electroplax. NPTMB causes a maximum depolarization of about 35 mV when applied to the dithiothreitol-treated sartorius motor endplate. It is ineffective on postjunctional membrane prior to disulfide bond reduction and on extrajunctional regions, reduced or unreduced. High concentrations of a competitive antagonist such as (+)-tubocurarine prevent reaction between NPTMB and the reduced receptor and cause a repolarization of the membrane when applied to the already-depolarized preparation. We conclude that in frog muscle, as in electroplax, the attached activator bridges the acetylcholine binding site of the reduced receptor between a sulfhydryl group, to which it is covalently bound, and a negative subsite, with which it forms a reversible ionic bond.

The acetylcholine (ACh) receptors of skeletal muscle and of the homologous electric tissue of electric fish convert the binding of ACh into a transient increase in the permeability of postsynaptic membrane to cations (Takeuchi & Takeuchi, 1960; Lassignal & Martin, 1977). These receptors, which are multichain, integral membrane proteins, have been purified and partially characterized (for reviews, *see* Karlin, 1977; Heidmann & Changeux, 1978).

The binding site of the nicotinic ACh receptor appears to be in close proximity to a readily reduced disulfide bond (Karlin, 1974). This disulfide bond is located about 1 nm from a negatively charged subsite that binds the quaternary ammonium moiety characteristic of receptor activators and inhibitors. One of the sulfhydryl groups liberated by chemical reduction is specifically affinity labeled by various quaternary ammo-

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nium alkylating or acylating agents. The sites of reaction of such affinity agents, radiolabeled, have been identified in receptors from *Electrophorus* electric tissue (Karlin & Cowburn, 1973), *Torpedo* electric tissue (Weill, McNamee & Karlin, 1974; Sobel, Weber & Changeux, 1977), and rat skeletal muscle (Froehner, Karlin & Hall, 1977).

Two types of affinity alkylating agents have been described. One type, exemplified by 4-(N-maleimido) benzyltrimethylammonium iodide (MBTA; Karlin, 1969), are covalently bonding inhibitors of the receptor. The other type, exemplified by the nitrophenyl ester of *p*-carboxyphenyltrimethylammonium iodide (NPTMB; Silman & Karlin, 1969), are covalently bonding activators. The site of reaction of the two types of affinity labels is the same (Damle, McLaughlin & Karlin, 1978). In the case of NPTMB, a trimethylammonium benzoyl (TMB) moiety is attached via a thioester bond to a receptor sulfhydryl, resulting in a depolarization that is not reversed by washing but is antagonized by high concentrations of reversibly binding competitive inhibitors such as (+)-tubocurarine (dTc) (Silman & Karlin, 1969). Covalently bound TMB apparently rotates about its point of attachment to the sulfhydryl group, interacting reversibly with the receptor anionic locus.

The exact mechanism by which ACh binding is linked to the postsynaptic membrane permeability increase is not yet understood. A recent and promising approach to the elucidation of the kinetics of synaptic gating mechanisms is the analysis of fluctuations in postjunctional membrane voltage or current during the steady exogenous application of ACh or other activators (for review, *see* Neher & Stevens, 1977). The statistical analysis of chemically-induced fluctuations at the frog neuromuscular junction and at extrajunctional regions of chronically denervated muscle fibers ideally can provide estimates of important single-channel parameters such as the mean lifetime and amplitude of the elementary permeability event (Katz & Miledi, 1970, 1971, 1972; Anderson & Stevens, 1973; Ben-Haim, Dreyer & Peper, 1975; Colquhoun *et al.*, 1975; Dreyer, Walther & Peper, 1976; Neher & Sakmann, 1976). Because, however, the rate-limiting step in the interaction of ACh with its receptor remains unidentified, it is not certain whether analysis of the time course of drug-induced fluctuations provides an accurate estimate of the mean open-channel lifetime or merely reflects the total lifetime of the drug-receptor complex.

We have approached the above question by employing an activator that attaches covalently near the receptor binding site. Because the quaternary ammonium group of covalently bound TMB is held within a molecu-

lar distance of the receptor anionic subsite, such an activator probably mimics the important limiting condition of saturating concentrations of a reversibly binding agonist. A necessary preliminary objective of the present work was therefore to demonstrate that the binding site of the ACh receptor of frog sartorius muscle bears sufficient structural similarity to its counterpart in *Electrophorus* electroplax to permit the covalent attachment of drugs originally developed and tested on electric tissue. Our first paper describes the reaction of NPTMB with the reduced sartorius receptor. The second paper presents an analysis of the voltage fluctuations induced by the covalently attached activator. A preliminary account of this work has been published (Cox, Karlin & Brandt, 1979).

Materials and Methods

All experiments were performed on junctional regions of frog (*Rana pipiens*) sartorius muscle fibers. The dissected preparation was routinely exposed to 1 mM dithiothreitol (DTT) (Sigma) in tris-buffered saline, pH 8.0, for periods of 20 to 45 min and then transferred to control saline of composition 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 0.425 mM NaH_2PO_4, 1.58 mM Na_2HPO_4 (pH 7.2).

The muscle was stretched to its *in situ* length and seated, ventral surface up, against the floor of a Lucite experimental chamber, which was in turn fixed to the stage of a Beck and Kassel compound microscope. A long-working-distance (14 mm) Leitz UM 20 objective lens permitted free movement of microelectrodes. Overall magnification was approximately 200. Temperature was maintained at 20–22 °C.

Capillary microelectrodes of outer diameter 0.8 mm were loaded with 3 M KCl for intracellular recording. Electrodes had tip resistance of 5 to 10 MΩ, measured in Ringer solution.

Acetylcholine (Sigma) was applied iontophoretically to junctional regions from microcapillaries containing drug concentrations of approximately 2.5 M. Electrode resistances ranged from 5 to 25 MΩ; holding currents of 5 to 10 nA usually proved sufficient. Current was supplied to the iontophoretic pipette by means of a current clamp designed by Dr. George Katz (Columbia University). Both current magnitude and electrode resistance were continuously monitored on a four-channel Tektronix Type 3A74 amplifier mounted in a Tektronix 561 A oscilloscope. Some iontophoretic pipettes displayed severe rectification and/or resistance fluctuations in response to an applied current and were therefore rejected. Other drugs (MBTA, NPTMB, dTc, etc.) were dissolved in unbuffered saline (pH ~ 6.0) and were applied by pressure release from microcapillaries of 3–10 μm tip diameter.

Experiments were performed only on junctional regions whose nerve supply could be seen to terminate on the surface of a fiber. Final positioning of the recording electrode was based on rise time and amplitude of observed miniature endplate potentials, as monitored on a Tektronix 564 storage oscilloscope. Rise times of about 1 msec and amplitudes of approximately 0.5 mV or greater were considered acceptable. No acetylcholinesterase inhibitors were used.

Results

NPTMB causes a large depolarization (about 35–40 mV, maximum) when applied from a pressure release capillary in the region of the DTT-

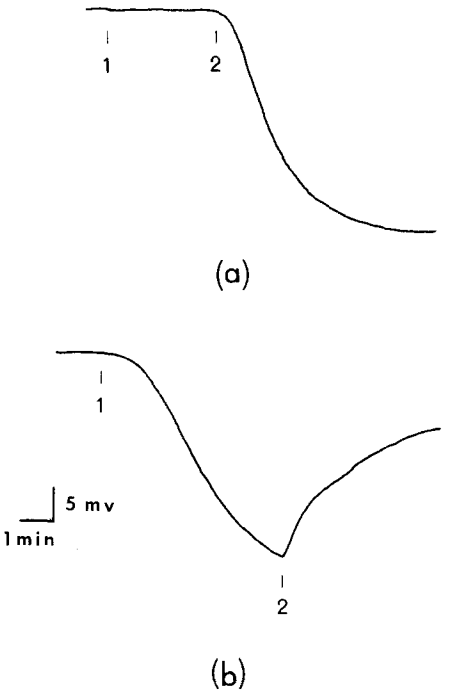

Fig. 1. Application of NPTMB to DTT-reduced frog sartorius neuromuscular junction. (*a*): 1 mM NPTMB in pressure release capillary. Original resting potential, 89 mV; maximum depolarization, 34 mV. Marker bars: *1*, beginning of application of drug; *2*, drug release terminated. (*b*): Initial resting potential, 88.8 mV. At marker bar *1*, the application of NPTMB, 100 μM concentration in the pressure release capillary, was initiated. Application was continued intermittently throughout the developing depolarization. At marker bar *2*, a second pressure release pipette containing 1 mM NPTMB was brought near the endplate. Release of drug at a high rate from both pipettes caused a rapid reversal of the depolarization, which at that point had reached 30.5 mV

reduced motor endplate. It has no observable effect when applied to unreduced endplates or to extrasynaptic areas, reduced or unreduced. With large concentrations of NPTMB ($\sim$1 mM) in the pressure release capillary, maximum depolarization does not develop until drug release is terminated (Figs. 1 *a* and 5). The same effect can be observed if smaller NPTMB concentrations ($\sim$ 50–100 μM) are loaded into the pressure release capillary, and the release is stepped up after a small depolarization has developed. Strikingly similar results have been reported upon bath application of NPTMB to the DTT-reduced electroplax and have been ascribed to a self-inhibitory action by the unreacted excess drug (Silman & Karlin, 1969).

The depolarization induced at the frog neuromuscular junction by covalently attached trimethylammonium benzoyl is not irreversible. Over

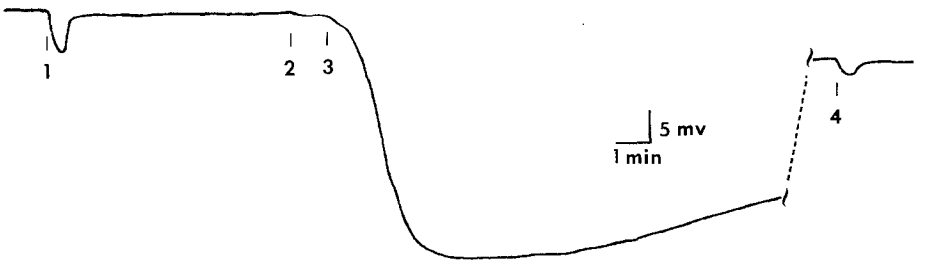

Fig. 2. Spontaneous recovery from TMB-induced depolarization. Initial resting potential 91.2 mV. At marker bar *1*, reducing the holding current across the ACh pipette to 0 produced a depolarization of 6.1 mV. Between marker bars *2* and *3*, NPTMB (1 mM in pressure release capillary) was applied for 45 sec. 45 min after reaching a maximum depolarization of 35 mV, membrane potential had recovered to within 8 mV of initial value. At marker bar *4*, application of ACh, +2 nA ejecting current, produced a depolarization of 2.2 mV. The break in the record covers 31.5 min

rather long periods of time after cessation of drug release ($\sim$ 1 hr), membrane potential will recover at least partially (Fig. 2). The covalently bound receptor appears to enter a desensitized (refractory) state. After the spontaneous reversal of the TMB depolarization, the receptor is insensitive to further application of NPTMB and shows a much reduced response to ACh (Fig. 2).

NPTMB is an active ester. As expected, prehydrolyzed NPTMB fails to react covalently with the binding site of the reduced ACh receptor (Fig. 3*a*). As an additional control, pure trimethylammonium benzoic acid, the quaternary ammonium hydrolysis product of NPTMB, was applied to several preparations, again without effect (Fig. 3*b*).

The quaternary ammonium maleimides 4-(N-maleimido) benzyltrimethylammonium (MBTA) and 4-(N-maleimido) phenyltrimethylammonium (MPTA) alkylate the reduced electroplax receptor 4700-fold and 2000-fold faster than does N-ethylmaleimide (Karlin, 1969; Karlin & Winnik, 1968). No depolarization is observed with MBTA; a small ($\sim$ 1 mV) depolarization is observed with MPTA. The reaction of both maleimide derivatives with the reduced receptor is impeded by a reversible receptor ligand such as hexamethonium. By the criteria of affinity enhancement and protectability (Singer, 1970), these quaternary ammonium maleimide derivatives are affinity labels of the reduced electroplax receptor binding site.

Solutions of 1 mM MBTA were applied from pressure release capillaries to sartorius neuromuscular junctions both before and after disulfide bond reduction. Neither depolarization by MBTA nor reduction of the

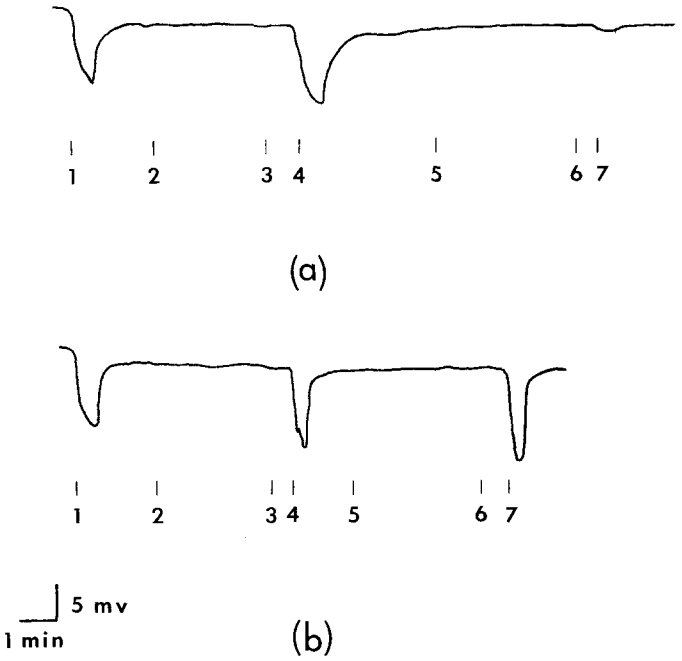

Fig. 3. Effect of hydrolyzed NPTMB, pure trimethylammonium benzoic acid, and MBTA on DTT-reduced sartorius endplate. (*a*): Original resting potential, 87.2 mV. Marker bars: *1*, response to ACh. Iontophoretic current, 0; depolarization, 10 mV. *2*, initiation of application of prehydrolyzed NPTMB, 100 μM concentration in pressure release capillary, pH 7.27. Release continued for 3 min and terminated at marker bar *3*. *4*, application of ACh. Iontophoretic current, 0 nA; depolarization, 10.2 mV. *5*, beginning of MBTA application, 1 mM concentration in pressure release capillary. Application period, 3.7 min; terminated at *6*. *7*, blockage of ACh response after exposure to MBTA. An iontophoretic current of +12 nA produced 0.9 mV depolarization. (*b*): Initial resting potential, 92.5 mV. At marker bar *1*, an iontophoretic current of +1 nA across the ACh pipette resulted in 10.3 mV depolarization. Pure trimethylammonium benzoic acid, concentration 1 mM in pressure release capillary, was applied twice – between marker bars *2* and *3* (3 min) and between bars *5* and *6* (3.5 min). At marker bars *4* and *7*, iontophoretic currents of +1 nA resulted in ACh depolarizations of 10.5 and 12.0 mV

ACh response was observed in the untreated preparation. In preparations previously exposed to DTT, MBTA application again produced no observable depolarization (or increase in voltage fluctuations), but the response to ACh was found to be blocked (Figs. 3*a* and 4).

Concentrations of (+)-tubocurarine sufficient to eliminate virtually all depolarization by ACh also protect the reduced sartorius receptor against covalent attachment of TMB; washout of the dTc restores sensitivity to both drugs (Fig. 5). Identical results were obtained in four other experiments of this kind.

Fig. 4. Effect of consecutive applications of MBTA. Initial resting potential, 83.5 mV. At marker bars *1*, *4*, *7*, and *10*, iontophoretic currents of +1.5 nA produced ACh responses of 10.0, 6.0, 3.7, and 1.4 mV. Interspersed with the ACh tests are 3 applications of MBTA, 1 mM in the pressure release capillary. *2–3*, 3-min application; *5–6*, 3.5-min application; *8–9*, 3-min application

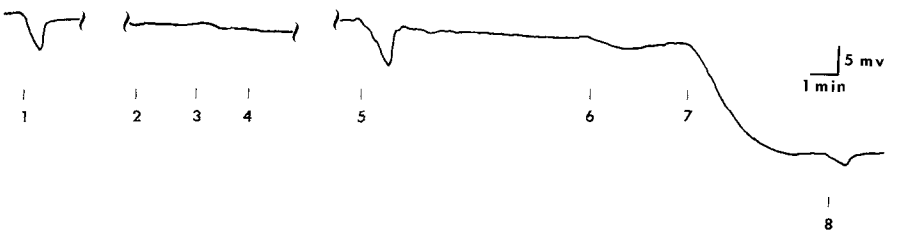

Fig. 5. Prevention of TMB binding by high concentrations of dTc. Original resting potential, 89.5 mV. *1*, test ACh response. Depolarization, 7.1 mV; iontophoretic current, +1.5 nA. The electrode was removed and 36 μM dTc was washed into the chamber. Break in record covers 10-min wash time. Resting potential after second penetration, 87.8 mV. At marker bar *2*, ACh was applied with an ejecting current of +5 nA. Application for 30 sec produced no depolarization. *3*, initiation of NPTMB release, 1 mM in pressure release capillary. *4*, termination of NPTMB release. During the 2-min application period and the 1.5-min following time, no effect was observed. Recording electrode was removed and control saline washed through chamber. Discontinuity in record covers 11-min wash period. Resting potential after third penetration, 88 mV. *5*, response to ACh. An iontophoretic current of +4 nA produced 9.0 mV depolarization. Release of NPTMB, begun at *6* and terminated at *7*, resulted in a depolarization of 21 mV. *8*, final ACh response. An iontophoretic current of +10 nA produced 2.2 mV depolarization

Application by pressure release of high concentrations of MBTA (Fig. 6) or dTc (Fig. 7) to the TMB-depolarized endplate causes a rapid membrane repolarization, as does exposure to an unreacted excess of NPTMB itself (Fig. 1*b*). Although these were the only three compounds tested for this effect, it might be inferred that any receptor ligand could compete with covalently attached TMB for the receptor binding site. Hence, at both the frog sartorius and the *Electrophorus* receptor, the covalently bound activator apparently rotates about its point of attachment to the SH group, the quaternary ammonium moiety interacting reversibly with the receptor anionic subsite (Silman & Karlin, 1969; Karlin, 1974).

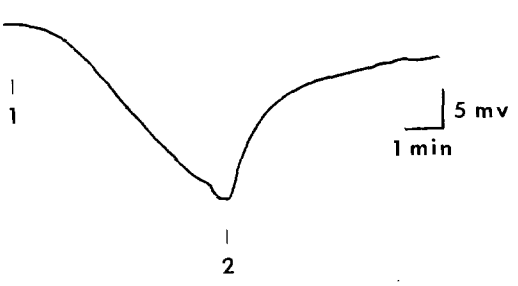

Fig. 6

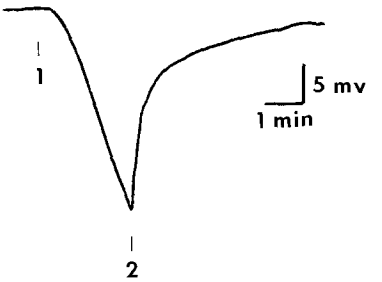

Fig. 7

Fig. 6. Reversal of TMB depolarization with MBTA. Initial resting potential, 89.3 mV. At marker bar *1*, release of NPTMB, 100 μM in pressure release capillary, was initiated. At *2*, a second pressure release capillary, containing 1 mM MBTA, was brought near the endplate, and the NPTMB pipette was withdrawn. Initiation of MBTA release caused a rapid membrane repolarization

Fig. 7. Reversal of TMB depolarization with dTc. Initial resting potential, 84.7 mV. *1*, initiation of NPTMB release, 100 μM in pressure release capillary. *2*, initiation of dTc release, 10 mM in pressure release capillary

Discussion

Covalent attachment of depolarizing groups to the ACh receptor depends upon the prior reduction of a disulfide bond near the receptor binding site. The effects on intact *Electrophorus* electroplax of disulfide bond reducing agents such as DTT have been documented extensively by Karlin and coworkers (Karlin & Bartels, 1966; Karlin & Winnik, 1968; Silman & Karlin, 1969; Karlin, 1969, 1973, 1974). Impairment of nicotinic ACh receptor response to monoquaternary activators following disulfide bond reduction has also been observed in leech dorsal muscle (Ross & Triggle, 1972), membrane vesicles from *Electrophorus* (Kasai & Changeux, 1971) and *Torpedo* (Schiebler, Lauffer & Hucho, 1977), *Aplysia* abdominal ganglion (Sato, Sato & Sawada, 1976), chick biventer cervicis muscle (Rang & Ritter, 1971), rat skeletal muscle (Albuquerque *et al.*, 1968), *Limnaea* parietal ganglion (Bregetovski *et al.*, 1978), and frog skeletal muscle (Albuquerque *et al.*, 1968; Mittag & Tormay, 1970; Del Castillo, Escobar & Gijon, 1971; Ben-Haim, Landau & Silman, 1973; Lindstrom, Singer & Lennox, 1973).

These studies have also revealed several species-specific differences. For example, the role reversal of the bisquaternary compound hexamethonium from weak antagonist to agonist that has been observed in the DTT-reduced electroplax has been confirmed in chick muscle, but not in leech muscle, frog muscle, or *Aplysia* ganglion cells. Also, the

acetylcholine analog bromoacetylcholine bromide apparently fails to produce in *Aplysia* or *Limnaea* cells the irreversible activation that has been observed (Silman & Karlin, 1969) in *Electrophorus* electroplax.

On a more detailed level, there are discrepancies regarding the precise location and role of the reducible disulfide bond. Lindstrom *et al.* (1973) have reported that neither MPTA nor MBTA blocks the reduced frog sartorius receptor more rapidly than does N-ethylmaleimide. Also, neither agonists nor antagonists retarded receptor alkylation. Lindstrom and colleagues concluded that the alkylated sulfhydryl function is located near, but is not a part of, the "active" site of the receptor. In contrast, Ben-Haim *et al.* (1973) found the reagent bromoacetylcholamine bromide to be a much more potent alkylating agent of the reduced sartorius receptor than is its sister compound bromoacetamide, which lacks the crucial quaternary ammonium. These authors argued that the reactive disulfide must be located a molecular distance from the anionic subsite. The apparent discrepancy between the results of these two studies may be at least partially ascribable to differences in technique and specific labeling agents used.

When applied to the reduced sartorius receptor, the covalently reacting agent NPTMB produces substantial depolarizations, as it does in electroplax. Hydrolyzed NPTMB causes no receptor activation. NPTMB is without effect on extrajunctional regions, with or without prior exposure to DTT. Also, application to the normal endplate is ineffective. Covalent attachment of TMB to the reduced receptor binding site is antagonized by concentrations of (+)-tubocurarine sufficient also to block the response to ACh. A reasonable interpretation of these data is that TMB binds to, or very near, the "active" site of the reduced receptor, interacting with one and the same anionic locus as does ACh. The investigations cited above and the results of the present study therefore support the conclusion that there are, at the very minimum, broad structural similarities in the nicotinic ACh receptor across an extensive phylogenetic range. This conclusion is further strengthened by the results of recent biochemical studies showing that MBTA is an affinity label of the solubilized, purified ACh receptor in species as widely separated as electric fish (Karlin & Cowburn, 1973; Weill *et al.*, 1974) and rat (Froehner *et al.*, 1977).

An intriguing feature of a covalently attached activator is that it may present to the receptor anionic subsite the equivalent of a very high concentration of reversibly binding drug. In theory, saturating drug concentrations can help to distinguish whether drug dissociation or iso-

merization of the drug-receptor complex is the rate-limiting step in the kinetics of interaction between agonists and the ACh receptor (*cf.* Stevens, 1975; Sheridan & Lester, 1975, 1977; Adams, 1977; Adams & Sakmann, 1978; Sakmann & Adams, 1978). It therefore seemed worthwhile to employ the technique of fluctuation analysis to investigate further the properties of the ACh receptor after covalent attachment of TMB. We describe the results of these experiments in the following paper.

This work is taken from a thesis presented by R.N. Cox in partial fulfillment of the requirements for the Ph.D. degree, Columbia University, October 1978.

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