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## Investigation of the Anticurare Effect of Sodium Fluoride at the Mammalian Skeletal Neuromuscular Junction: A Dissertation

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INVESTIGATION OF THE ANTICURARE EFFECT  
OF SODIUM FLUORIDE AT THE MAMMALIAN  
SKELETAL NEUROMUSCULAR JUNCTION

A dissertation presented by  
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for the degree  
of  
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## ABSTRACT

The anticurare action of sodium fluoride was investigated in isolated cat tenuissimus muscle utilizing intracellular microelectrode techniques. Analysis of the data from these experiments resulted in the following findings:

- 1) A postsynaptic sensitization phenomena was demonstrated on the basis of an increased amplitude of the miniature end plate potential.
- 2) Evidence for a presynaptic site of action of fluoride was presented.
- 3) The anticurare action of fluoride is realized by increasing the amplitude of the end plate potential under conditions in which the miniature end plate potential does not increase in amplitude.
- 4) The increased end plate potential amplitude is due to a greater number of quanta being released from the nerve terminal.
- 5) Fluoride produced an increase in the number of quanta in the readily releasable pool by increasing mobilization of quanta from another transmitter source.
- 6) Since the probability of release did not change, the number of quanta released increased by virtue of the increased size of the readily releasable

ble pool of quanta.

7) It was pointed out that, although fluoride is antitumor in both the frog and mammal, there appeared to be differences in its mechanism of action.

## BIOGRAPHY

Robert S. Jacobs was born on April 2, 1933, in Chicago, Illinois. He graduated from South Shore High School and continues his studies at Wilson Jr. College. He received the Bachelor of Philosophy degree in biology from Northwestern University in 1964.

From 1957 to 1967 he was an employee in the department of pharmacology of G.D.Searle & Company where he served as supervisor of their pharmacology screening laboratories.

Graduate training in the department of pharmacology at Loyola University Medical Center was begun in 1965 under Dr. L.C.Blaber.

Mr. Jacobs was supported by a predoctoral training grant and research grant of the National Institutes of Health from 1967 to 1970. In July, 1970, he was appointed research associate in the department of pharmacology.

On September 7, 1957, Mr. Jacobs was married to Joan Elizabeth Rossdeutcher. A daughter, Rebecca, was born June 13, 1962.

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## ABBREVIATIONS

ACH	Acetylcholine
ADP	Adenosine diphosphate
AgCl	Silver chloride
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
(C <sub>10</sub> )	Decamethonium
Ca	Calcium
Cl	Chloride
cm	Centimeter
CO <sub>2</sub>	Carbon dioxide
(c.v.)	Coefficient of variation
DCI	Dichloroisoproterenol
DNP	2:4 Dinitrophenol
ECG	Electrocardiogram
EMP	Embden-Meyerhof pathway
e.p.p.'s	End plate potentials
gm	Gram
HMP	Hexose monophosphate shunt
K	Potassium
KCl	Potassium chloride
Kg	Kilogram
K <sub>i</sub>	Potassium iodide
(m)	Quantal units in an e.p.p.
m.e.p.p.'s	Miniature end plate potentials
meq/L	Millequivalent per liter
Mg	Magnesium
[Mg][F] <sup>2</sup> [PO <sub>4</sub> ]	Magnesium fluorophosphate
mg/Kg	Milligram per kilogram
ml	Milliliter
mm	Millimeter
msec	Millisecond
mV	Millivolt
n	Total number
NaF	Sodium fluoride
NH <sub>3</sub>	Ammonium
O <sub>2</sub>	Oxygen
(p)	Average probability
2-PAM	Pyridine-2-aldoxime methiodide
(P <sub>2</sub> S)	N-methylpyridinium-2-aldoxime methane sulphate
q	Quantal size
R.P.	Resting potential
S <sub>2</sub>	Standard deviation
(SCh)	Succinylcholine
u	Micron
ug/ml	Microgram per milliliter

## GLOSSARY

<u>Term</u>	<u>Definition</u>
exp.	Exponential
T	Time
n	Number
p	Probability
M	Quantal content
N	Readily releasable store
dm	Mobilization of quanta
q	Quantal size
Train	A series of end plate potentials. e.q. 100 e.p.p.'s in a train.
Head	The first few e.p.p.'s in a train.
Tail	Those e.p.p.'s remaining after the initial first few e.p.p.'s (head).
frequency	Number of stimuli or response per second.
stimulus interval	Interval between pulses (msec).



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## ANATOMY

The cat tenuissimus muscle is a long, slender, white muscle located in the hind leg of the cat. It arises at the level of the transverse process of second caudal vertebra and extends laterally along the inner surface of the popliteal space, terminating in the connective tissue of the underlying biceps femoris muscle.

The motor nerve supplying the tenuissimus arises from the sciatic as a small bundle extending peripherally for a few centimeters as a trunk of fibers. As it approaches the lower  $\frac{1}{3}$  of the muscle, the nerve trunk divides into two branches which then enter the connective tissue of the muscle and travel distally to terminate at opposite ends of the muscle. As each main nerve branch courses the length of the muscle, it divides many times to send out fibers laterally and, thus, innervate various segments of the muscle mass. In most cases a secondary fiber arises from one or both of the two main branches just before entering the muscle mass to innervate a small central area of muscle lying between the bifurcation of the main trunk and the points where the two main branches enter the muscle. The many nerve endings of each nerve

fiber are distributed randomly, so that a single motor unit contributes to contraction in numerous locations along the length of the muscle.

Estimates indicate there are 50-100 nerve fibers within each of the two main nerve branches. The sum of the fibers in each branch is always more than is found in the initial trunk indicating that individual fibers divide before the nerve trunk bifurcates and enters the muscle. It is not presently known if the secondary branches innervating the small central portion of the muscle are composed of more than a single nerve fiber (Brown, 1913; Adrian, 1925; Denny-Brown, 1929; and Cooper, 1929).

The intact muscle mass is about 9-15cm long and 2-4mm wide. A cross section of tenuissimus muscle usually contains approximately 700-1300 fibers of average diameter approximating 30u. The surface cells average 35u. The average length of each cell is 17.3mm, and extremes of 13mm and 25.5mm have been observed. The individual muscle fibers taper gradually and end in long pointed processes which are organized throughout the muscle mass in an interdigitating form.

Motor end plates of tenuissimus muscle have been generally believed to conform to descriptions for other types of mammalian end plates. Electron microscopy

of tenuissimus neuromuscular junction has not been reported in the literature, however electrophysiological evidence supports the probability that a chemically sensitive subsynaptic membrane is present. Presynaptic elements are evidenced by the presence of miniature end plate potentials (m.e.p.p.'s). Evidence of the presence of a specialized synaptic membrane on the muscle can be indicated by the end plate potential (e.p.p.'s) attenuation occurring when measuring e.p.p.'s at various distances from a focus of maximum electrical activity in the same cell (Martin, 1955).

Certain types of information descriptive of the anatomy of the neuromuscular junction of the cat tenuissimus muscle are not available. For example, visualization of vesicles in the motor nerve terminal as described for other muscle (Birks, Huxley and Katz, 1960; Hubbard and Kwanbunbumpen, 1968), junctional folds (Palade and Palay, 1954; Reger, 1954; and Robertson, 1954), or a synaptic trough (Anderson-Cedergren, 1959; and Deharven and Coers, 1959) have not been described for tenuissimus muscle. Similarly absent are measurements of the synaptic cleft (Palay, 1958).

From an anatomical point of view, it would appear that considerable investigation into the innervation and structure of the neuromuscular junction in the

cat tenuissimus muscle could provide useful and interesting information.

## PHYSIOLOGY

Mechanical properties of tenuissimus muscle. -

The cat tenuissimus is a flexor-reflex muscle that contracts simultaneously with the posterior part of the biceps femoris (Sherrington, 1910). Graham Brown (1913), in measuring grades of muscle contraction in response to various intensities of a reflex induced stimulus, concluded that the tenuissimus efferent motoneurons and muscle fibers do not respond in an "all or none" fashion. He based his conclusion on the observation that there are a greater number of different mechanical responses occurring after reflex stimulation than the number of efferent fibers present in the motor nerve. In contrast, Porter and Hart (1923) demonstrated in tenuissimus muscle that contraction amplitude proceeds in step-like increments as the shock intensity increases in strength. Each step-like increment occurs as an "all or none" event. They also demonstrated the same step-like increments are obtained when the muscle is made to contract by reflex stimulation. Based on the variation in amplitude of muscle contractions obtained, they suggested that it was unlikely in the case of the tenuissimus muscle that each motoneurone innervates the same number of muscle fibers.

These authors indicated that one increment of contraction may represent the activity of 40 muscle fibers, another that of 10 fibers, and so on.

Adrian (1925) demonstrated by direct stimulation studies and histological examination that 1) the tenuissimus muscle fibers were much shorter than the length of the muscle, and 2) localized contractions occurred in the immediate vicinity of an electrode applied directly to curarized muscle. The localized contractions occurring were not due to decremental conduction along a single fiber but to the "all or none" contraction of several small muscle fibers.

In the same series of experiments, Adrian (1925) reported that both halves of a transversely cut tenuissimus muscle contracted when the nerve trunk was stimulated, indicating that muscle contraction was elicited by nerve elements innervating each half of the muscle, and electrical conduction from one muscle fiber to the next was not required. This investigator further demonstrated that some of the nerve fibers in the two main branches innervating the tenuissimus are formed from a single parent fiber in the main nerve trunk. This was made obvious by the fact that contractions in a transversely sectioned muscle could be elicited in both halves of the preparation by stimula-

tion of either nerve branch.

Cooper (1929) extended Adrian's work. His data indicate that motor unit distribution in tenuissimus muscle was in a chain like fashion rather than side by side. Therefore, in a fractional contraction elicited by a weak stimulus, both halves of the muscle contract, and each half contracts throughout its length. A large contraction means that more parallel chains are brought into action.

It can be summarized that the individual muscle fibers of cat tenuissimus contract in an "all or none" fashion, and motor units of the muscle are distributed in a chain like sequence. Submaximal stimulation elicits fractional contractions due to stimulation of only a few motor units. Increasing stimulation intensity increases the number of motor units excited.

From a functional point of view, skeletal muscles have been classified into slow, tonic muscles used, for example, to maintain posture, and fast, twitch muscle used for rapid movement such as writing or walking. Classification of muscle as fast or slow is determined by contraction time and the presence of red or white fibers in the muscle. Examples of these differing muscle types are found in the soleus muscle and tibialis muscle of the cat. Soleus muscle



is considered a slow muscle, because its contraction time is slow, and because it contains primarily red muscle fibers. Tibialis muscle, on the other hand, as well as tenuissimus muscle are considered fast twitch muscles (MacLagan, 1962), because contraction time is fast, and the fibers are primarily white. There is an additional classification of skeletal muscle. This is based essentially on the identification of slow tonic muscles in amphibians, birds, and mammals which are innervated and behave differently than other types of skeletal muscle. These slow amphibian muscles receive multiple nerve endings, have no clearly defined end plates, and do not exhibit action potentials. In addition, they require multiple stimulation before they contract. Twitch muscles, on the other hand, are innervated by single unmyelinated motor nerve terminals, have clearly defined end plates, elicit action potentials, and contract in response to a single stimulus.

The cat tenuissimus muscle is composed of primarily white fibers (MacLagan, 1962), responds to a single stimulus (Adrian, 1925), and has a peak contraction time of 30msec with duration of 100msec (MacLagan, 1962). Thus, it is quite similar to the tibialis muscle of the cat (contraction time 20msec, duration 80msec).

Another feature of fast twitch muscle is the character of its response to tetanic stimulation. If one applies repeated indirect stimulation to the muscle, the muscle fibers summate resulting in a successive series of contractions which fuse. The soleus muscle of the cat has a fusion frequency of about 30 shocks per second, tibialis muscle has a fusion frequency of about 100 shocks per second. Maclagan (1962) has reported that the cat tenuissimus muscle has a fusion frequency identical to that of the tibialis muscle. It can be assumed from these data that the tenuissimus muscle behaves physiologically as a fast twitch muscle. Electrophysiological studies have shown that action potentials can be elicited in this preparation (Boyd and Martin, 1956b). It should be pointed out, however, that it is possible that this muscle may contain other types of muscle fibers as well (Adrian, 1925). It is not known if these other fibers are slow tonic fibers or not. Further, it is yet to be determined by microscopy if the individual fibers of tenuissimus muscle are innervated by single motoneurons.

A number of important properties of mechanical muscle contraction are yet to be measured in cat tenuissimus muscle. The latent period between an action potential and the onset of contraction has not been

measured. Latency relaxation, resting length, and tension relationships, as well as contribution of elastic components to contraction in this muscle, have not been determined. Similarly, data on heat of contraction are as yet unavailable.

Electrophysiological properties of tenuissimus muscle. - At many synapses the evidence for the quantal theory of transmitter release rests in part on the observation that spontaneous m.e.p.p.'s occur. Analysis of electrophysiological nature of synaptic transmission derived from data obtained through the utilization of intracellular microelectrode methods and certain correlations drawn from topographical and anatomical features of the synapse have led to the contemporary theory of neuromuscular transmission.

In brief, a nerve action potential depolarizes the nerve terminal (Katz and Miledi, 1966). The depolarization produced in this manner in the unmyelinated segment of the terminal results in an influx of Ca ion which in turn, by a mechanism as yet not understood, releases quanta of ACh from nerve terminal storage sites (Hubbard and Schmidt, 1962; and Katz and Miledi, 1965c). The released quanta diffuse across the synaptic gap and combine with receptors in a special chemically

sensitive subsynaptic region of the muscle fiber. The chemical interaction of transmitter with muscle end plate receptors results in the summation of a series of local conductance changes (Katz and Theslauff, 1957). The value of each local conductance change is dependent on the size of each ACh quantum (molar strength), the density of the receptor sites, and the input resistance of the muscle fiber (Fatt and Katz, 1951, 1952; and Takeuchi and Takeuchi, 1960).

It can be assumed that the number of receptors reacting is dependent upon the number of quanta released. If a sufficient release of quanta occur, the summated conductance changes produced are sufficient to excite the electrically sensitive portion of the muscle membrane and elicit a muscle action potential which is propagated along the surface of the muscle. The consequence of all of these events leads to an "all or none: shortening of the muscle fiber by activating the action potential-contraction coupling mechanism.

Evidence supporting this concept of neuromuscular transmission rests upon three types of electrophysiological information and on parallel data dealing with anatomical and topographical relationships between the nerve terminal and muscle fiber. Specific data for

cat tenuissimus muscle are absent in some cases. However, the available evidence tends to support what has already been determined in other species of animal and other muscle types (Martin, 1955; Blaber and Christ, 1967). The three types of electrophysiological evidence are:

- 1) Investigations of m.e.p.p.'s.

- 2) The quantal nature of e.p.p.'s.

- 3) The pharmacological action of certain relatively specific drugs.

The visual evidence regarding nerve-muscle organization has been obtained through development of electron microscopic techniques.

M.e.p.p.'s recorded by microelectrode methods have been detected in mammalian twitch fibers (Boyd and Martin, 1956b; Brooks, V.B., 1956; Elmqvist, Hofmann, Kugelberg and Quastel, 1964; and Liley, A.W., 1956), in birds (Ginsborg, 1960), in fish (Takeuchi, 1959), in slow fibers of frogs (Burke, 1957), in mammals (Hess and Pilar, 1964), and in mammalian smooth muscles (Dudel and Kuffler, 1961; and Usherwood, 1963). They were first discovered in frog muscle by Fatt and Katz (1952).

Determination of the spontaneous nature of m.e.p.p. release depends largely on mathematical treatment of the phenomenon. If the discharge from nerve terminals

is random in nature, there are several inferences inherent in this event that shed some light on the release mechanism. Fatt and Katz (1952) found that the cumulative distribution of m.e.p.p. intervals was exponential and followed the formula:

$$n = N(1 - \exp^{-t/T})$$

Thus, the intervals between m.e.p.p.'s were found to be independent of one another. This can be explained as a threshold level of an "all or none" event. In other words, each m.e.p.p. cannot be further divided. This condition is important in assessing the quantal nature of transmitter release in that it relates random release with time. Observation of a single m.e.p.p. either occurs or does not occur in time, and each occurrence is independent of the previous one or the next one (Burnstock and Holman, 1962; and Martin, 1966). Martin (1966) and Fatt and Katz (1952) pointed out that, even if the discharge is completely random, the frequency distribution of m.e.p.p.'s could be composed of a large number of independent units discharging at regular intervals. Extracellular recordings from "active spots" have minimized this possibility, since it was reported that individual units themselves discharge in an irregular manner (Del Castillo and Katz, 1956; Dudel and Kuffler, 1961; and Fatt and Katz, 1952).

It has been well established that the spontaneous occurrence of m.e.p.p.'s are distributed in a manner predicted by the Poisson formula (Fatt and Katz, 1952; and Boyd and Martin, 1956b). If this is true, the release of any one quanta should not significantly alter the probability of release of remaining quanta (Gage and Hubbard, 1965).

A number of factors appear to affect spontaneous release. Increasing  $\text{Ca}^{++}$  and increasing  $\text{K}^+$  are two of the widely studied ions. Gage and Hubbard (1965) found that when the nerve terminal is depolarized by bathing the muscle in KCl the very large increase in m.e.p.p. frequency remains predictable by the Poisson formula.

M.e.p.p. release is also altered by other factors such as osmotic gradient (Hubbard, Jones and Landau, 1968a,b),  $\text{Ca}^{++}$  concentration (Hubbard, Jones and Landau, 1968a,b), stretch (Fatt and Katz, 1952), temperature (Fatt and Katz, 1952; Boyd and Martin, 1956b; Liley, 1956a; and Hubbard, Jones and Landau, 1967), and pH (Hubbard, Jones and Landau, 1968a). Boyd and Martin (1956b) found that doubling the tonicity of the bathing solution increased m.e.p.p. frequency in cat tenuissimus muscle. This osmotic effect was studied in detail in rat diaphragm preparations by Hubbard, Jones and Landau (1968a,b). They concluded that m.e.p.p. frequency can

be increased by more than one mechanism, and the effects of osmotic gradients on m.e.p.p. frequency were independent of effects due to  $\text{Ca}^{++}$  or depolarization of nerve terminals.

$\text{Ca}^{++}$  ions have been shown to be intimately involved in transmitter release. Increasing  $\text{Ca}^{++}$  concentration results in increased m.e.p.p. frequency, while decreasing  $\text{Ca}$  concentration decreases m.e.p.p. frequency (Boyd and Martin, 1956b; Jenkinson, 1957; Hubbard, 1961; and Hubbard, Jones and Landau, 1968a,b).  $\text{Mg}$  ions do not alter m.e.p.p. frequency in cat tenuissimus muscle. Raising temperature increases m.e.p.p. frequency; lowering temperature decreases m.e.p.p. frequency (Fatt and Katz, 1952; and Boyd and Martin, 1956b).

The role of  $\text{Na}^+$  ions on m.e.p.p. release has not been studied in cat tenuissimus muscle. Birks (1963) from his studies in rat diaphragm has found that accumulation of "intraterminal"  $\text{Na}^+$  results in increased m.e.p.p. frequency. In the presence of elevated  $\text{K}^+$ , reductions in  $\text{Na}^+$  lead to marked increase in m.e.p.p. frequency (Gage and Quastel, 1966). Since lowering  $\text{Na}^+$  will consequently reduce the amplitude of the pre-synaptic action potential, it has been suggested that the facilitatory effect of decreased  $\text{Na}^+$  is counter-acted by depression of the release mechanism (Gage and



Quastel, 1966; and Hubbard, Llina's and Quastel, 1969).

Liley (1956) attempted to relate m.e.p.p. frequency to quantal content of an e.p.p. The a priori condition of this hypothesis rests on a correlation of the effects of nerve terminal depolarization on m.e.p.p. frequency, and the fact that invasion of the nerve terminal by an action potential is a depolarizing event. Therefore, it is suggested that the depolarization caused by an action potential would increase m.e.p.p. frequency in an exponential fashion. The recruited m.e.p.p.'s would thus form the unit components of the e.p.p. (Liley, 1956). Although this hypothesis is largely still valid today, the mechanism proposed by Liley, Birks, and others had to be revised.

Using ionophoretic application of  $\text{Ca}^{++}$  applied to nerve terminals, Katz and Miledi (1966) found that by synchronizing the arrival of a nerve impulse with a brief pulse from a  $\text{Ca}^{++}$  electrode a facilitatory effect on transmitter release could be produced after the action potential wave had already reached its peak.

These data indicate that the process of transmitter release continues to operate during the falling phase of the nerve action potential, thus suggesting that nerve terminal depolarization may activate a more complicated mechanism that to release transmitter directly.

Utilizing tetrodotoxin to prevent impulse propagation, these same investigators advanced our understanding of transmitter release with the following findings:

1) Depolarization of motor nerve endings by locally applied current still produced an increased frequency of m.e.p.p.'s.

2) When brief current pulses were applied, e.p.p.'s evoked varied with the current intensity and were shown to be composed of a statistically varying number of m.e.p.p.'s.

3) The responses failed when  $\text{Ca}^{++}$  was removed.

4)  $\text{Na}^+$  ion which was prevented from entering the nerve by tetrodotoxin was not essential for transmitter release.

Using this same method, they further found that the release of ACh lags behind the depolarization. In fact, if very brief duration pulses are used, the release starts after the end of the pulse. Lengthening the pulse increases the rate of transmitter release and also increases the latency.

The evidence that external  $\text{Ca}^{++}$  acts directly on the release mechanism (Hubbard and Schmidt, 1963; and Katz and Miledi, 1965c) suggests that the inward movement of  $\text{Ca}^{++}$  is a critical step in the process of

"stimulus-secretion coupling". It appears, therefore, that the surface negative pulse (nerve action potential) raises the membrane permeability to  $\text{Ca}^{++}$  ions. After a brief delay,  $\text{Ca}^{++}$  ions enter the nerve terminal and release transmitter quanta. The number of quanta released per  $\text{Ca}^{++}$  ion is not positively known at present. Estimates vary from 3 or 4 quanta (Dodge and Rahamimoff, 1967) to several (Katz and Miledi, 1967).

In 1954 Del Castillo and Katz made a study of m.e.p.p.'s occurring in frog muscle and their relation to the e.p.p. response. It was previously suggested that the e.p.p. is built up statistically of small "all or none" units which are identical in size with the spontaneous m.e.p.p.'s. This evidence was developed from experiments in which the quantum content of the e.p.p. had been reduced to a small number by lowering the external  $\text{Ca}^{++}$  ion (Fatt and Katz, 1952). Under these conditions, the size of the e.p.p. approached that of the m.e.p.p. This observation was confirmed by Del Castillo and Engback (1954) using  $\text{Mg}^{++}$  rich bathing solution and in cat tenuissimus muscle by Boyd and Martin (1956b). At a certain level of low  $\text{Ca}^{++}$  or high  $\text{Mg}^{++}$  "all or none" fluctuations are observed in response to successive nerve stimulation. In other words, a point of reduction in quantal release is reached

where a nerve impulse may or may not release a single quanta.

There is as yet some question whether quantal release by nerve impulses are an acceleration of spontaneous release, or whether spontaneous and evoked release occur by different mechanisms. Del Castillo and Katz (1954) observed the occurrence of m.e.p.p.'s in frog muscle in both the presence of high  $Mg^{++}$  and in the absence of  $Ca^{++}$  ions. In mammalian preparations however, m.e.p.p.'s appear to be  $Ca^{++}$  mediated (Boyd and Martin, 1956b; Elmqvist and Feldman, 1965a; Hubbard, 1961; and Hubbard, Jones and Landau, 1968a). There is a release component however which is not  $Ca^{++}$  dependent (Hubbard, et.al., 1968a). It should be pointed out that alterations in m.e.p.p. frequency do not predict altered release by a nerve impulse. Changes in m.e.p.p. frequency may come about by other mechanisms that precede transmitter release. On the other hand, statistical evidence does support certain similarities between m.e.p.p.'s and nerve impulse evoked release. In low  $Ca^{++}$  or high  $Mg^{++}$  the partially-blocked release mechanism in mammalian muscle responds to nerve stimulation in an "all or none" fashion predictable by the Poisson formula. The frequency of m.e.p.p.'s can also be predicted by this equation. This suggests that both

spontaneous release and nerve impulse release are of a quantal nature. What the Poisson formula does not reveal is whether evoked quantal release comes from the same source as the m.e.p.p.'s.

From these considerations a quantal hypothesis of transmitter release has evolved. This concept has led to the identification of a system in the nerve terminal whose function is to control storage and release of transmitter.

If a population of quanta are stored in the nerve terminal, the total number being given by the value  $n$ , the average probability ( $p$ ) of responding to a single nerve impulse is given by relation  $m = np$ , where  $m$  = the number of quanta released. Under normal conditions,  $p$  is relatively large (i.e. a large part of the population responds to a nerve impulse). When  $\text{Ca}^{++}$  is lowered (or  $\text{Mg}^{++}$  increased),  $p$  becomes smaller and smaller until complete failure occurs.

Under these conditions, when  $p$  is small, the number of quantal units ( $m$ ) which make up the e.p.p. should be distributed according to the Poisson formula:

$$\frac{\exp (-m)m^x}{x!}$$

An alternative way of determining  $m$  (the number of quanta in an e.p.p.) is to utilize the relation

of mean e.p.p. amplitude and of mean m.e.p.p. amplitude. This is based on the assumption that an e.p.p. is composed of a certain number of m.e.p.p.'s. Thus:

$$m = \frac{\text{mean amplitude of e.p.p.}}{\text{mean amplitude of m.e.p.p.}}$$

This equation depends on the assumption that there is linear summation of the m.e.p.p. components of the e.p.p.'s. This is true if the e.p.p. is relatively small compared to the resting potential (Del Castillo and Katz, 1954).

A second measurement of quantal content can be formulated from the Poisson statement:

$$\frac{\exp(-m)m^x}{x!}$$

When  $x = 0$  (the number of responses containing zero quanta)

$$m = \log_e \frac{\text{number of nerve impulses}}{\text{number of failures of e.p.p. response}}$$

These formulas are equivalent:

$$m = \frac{\text{e.p.p. amplitude}}{\text{m.e.p.p. amplitude}}$$

$$\log_e \frac{\text{number of stimuli}}{\text{number of failures}}$$

This second estimate of quantal release has been repeatedly demonstrated to result by decreasing extra-

cellular  $\text{Ca}^{++}$  or increasing  $\text{Mg}^{++}$  (Del Castillo and Katz, 1954).

A third measurement of quantal content arises from a property of the Poisson distribution is that in this case the standard deviation of a series of measurements is equal to the square root of the mean. If the quantum contents of individual synaptic potentials in a series are distributed according to the Poisson equation, then coefficient of variation (c.v.) of the quantum content distribution should be equal to  $1/\sqrt{m}$ . Since the coefficient of variation is independent of the unit of measurement, the coefficient of variation of the amplitude distribution should be identical with the coefficient of variation of the quantum content distribution, except that allowance must be made for the fact that the unit potentials (m.e.p.p.'s) themselves are not of uniform size (Martin, 1966). Therefore:

$$\text{c.v.} = \frac{1}{\sqrt{m}}$$

making allowance for the variation in unit size,

$$\text{c.v.} = \frac{1 + \text{c.v.'}}{\sqrt{m}} \quad m = \frac{1 + (\text{c.v.'})^2}{(\text{c.v.})^2}$$

where c.v'. = the coefficient of variation of the unit potentials.

It has been reported by numerous investigators

that c.v'. is usually less than 0.05 and in practice has been ignored (Blackman, Ginsborg and Ray, 1963; Boyd and Martin, 1956b; Dudel and Kuffler, 1961; Edwards and Ikeda, 1962; and Fatt and Katz, 1952).

All three methods of estimating quantal content are useful only when the e.p.p. amplitudes are of small size relative to the resting potential.

To accomplish this, the amplitudes of e.p.p.'s had to be reduced by reduction of  $\text{Ca}^{++}$  ions in the bathing solution, by increasing  $\text{Mg}^{++}$ , or by blocking the muscle with d-tubocurarine (Del Castillo and Katz, 1954; and Boyd and Martin, 1956b). It was found that when  $m$  was greater than about 10, the amplitudes of the e.p.p.'s were more compressed on the voltage scale than expected, and the coefficient of variation was generally less than expected. It was suggested by Del Castillo and Katz that non-linear summation contributes significantly to the discrepancy between measurements of c.v. and the proposed theoretical  $1/\sqrt{m}$  (Martin, 1955).

Non linear summation is based upon the principle that as the end plate depolarization proceeds towards equilibrium potential, the contribution of each quantum to the e.p.p.'s become less and less. Under these conditions, the voltage scale is flattened, and the coefficient of variation decreased. This "non-line-



arity" of response can be corrected when allowance is made for the non-linear relation between conductance and potential changes.

$$\text{E.P.P.} = \frac{\text{E.P.P.} \times (\text{R.P.} - 15)}{\text{E.P.P.} - \text{R.P.} - 15}$$

This equation (Martin, 1955) was found to correct e.p.p. amplitude in a manner that restores the relation between mean quantum content and coefficient of variation for e.p.p. values exceeding 5mV.

This correction allowed fuller utilization of the method to examine mathematically the quantitative aspects of transmitter storage and release in the absence of conditions which affect the very mechanism under study (e.g. high  $\text{MgCl}_2$  or decreased  $\text{CaCl}_2$  in the bath).

Still required was the use of agents that prevent muscle movement. For example, it is common practice, when utilizing microelectrode methods, to block the muscle with d-tubocurarine. This agent is known to antagonize competitively ACh postsynaptically and thus reduce the amplitude of e.p.p.'s below threshold. Under these conditions, the contribution of each quanta to the e.p.p.'s is reduced uniformly, and one is able to obtain an estimate of quantal content from the coefficient of variation of a train of e.p.p.'s.

When a d-tubocurarine blocked muscle is stimu-

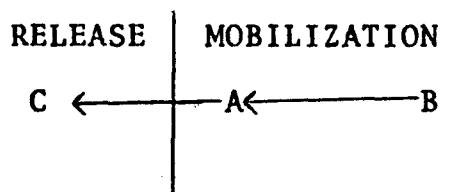
lated at a sufficient frequency, the succeeding e.p.p.'s in a train are reduced in amplitude for approximately the first 10 responses, after which the train levels off then recedes at a much slower rate. The initial e.p.p. decline appears to be due to depletion of the transmitter from a readily releasable store (Liley and North, 1953). The amplitudes of the declining e.p.p.'s are assumed to be proportional to declines in quantal content.

A possible objection to the above assumption is that some of the depression may be due to postsynaptic desensitization. Experiments with iontophoretic application of ACh have shown this phenomenon to occur (Katz and Thesleff, 1957). Thesleff (1959) reported some desensitization at mammalian end plates, but experiments by Otsuka, Endo and Nonomura (1962) could not substantiate this. These authors found that the depression was increased by increasing the  $\text{Ca}^{++}$  concentration of the bathing solution but was unaffected by anti-ChE's agents. In other words, the regression of e.p.p.'s appears to be the result of decreasing quantal release rather than desensitization (Martin, 1966; and Brooks and Thies, 1962). Elmqvist and Quastel (1965) analyzed a number of experiments in human neuromuscular preparations. They found that quantal size

remained constant during a series of 10 sequential tetanic trains. When the stimulus frequency was increased up to 200/second, quantal size increased. This was not consistent with desensitization where quantal size should decrease.

The fact that the rate of decline in e.p.p. amplitude levels off after a few end plate responses is evidence of replacement of available quanta, as they are released (Martin, 1966; and Elmqvist and Quastel, 1965). These data indicate that only part of the ACH stores present is in a readily releasable state.

Still another assumption required for quantitative study of e.p.p.'s is that the early tetanic run down (i.e. decline in e.p.p. amplitude) is due to depletion of presynaptic stores which are readily releasable (Liley and North, 1953; and Otsuka, Endo and Nonomura, 1962). At the point where the rate of decline changes (plateau), it must be concluded that the immediately available store was supplied with new material (i.e. there was mobilization of transmitter to become available for release) (Elmqvist and Quastel, 1965). These investigators carried the analysis further, by quantitatively assessing the relationship between the readily releasable store (A) and the store which fills it (B). Their hypothesis is represented as follows:



Only data for frequencies above 60/second fit their hypothesis. At lower frequencies, the relationship between differences in concentration in stores and stimulus frequency became non linear.

At the present time, it is felt that mobilization is somewhat delayed, and, under conditions where large depletion from the available store occurs, the amount of transmitter released falls below the level at which mobilization later sustains it. Hence, there is an initial rapid transmitter depletion in a train of pulses followed at some point in time by a leveling off of the height of each end plate response which is sustained by presynaptic mobilization from a less readily releasable store (Liley and North, 1953; Hubbard, 1963; and Elmqvist and Quastel, 1965). Proceeding from this point, one finds another regression in end plate response that proceeds much more slowly. This second regression is believed to be due to gradual depletion of the secondary less readily releasable store (Martin, 1955; Liley, 1956b; and Elmqvist and Quastel, 1965).

Because of the rapid depletion in readily releasable stores with high frequency stimulation, it became possible to estimate fractional release and the store size from the following relation:

$$p = \frac{m}{n}$$

where  $p$  = probability of release;  $m$  = quantal content;  $n$  = readily releasable stores (Elmqvist and Quastel, 1965; and Martin, 1966).

Quantal content of each e.p.p. during the early depletion is calculated from the coefficient of variation in the last 80 or so e.p.p.'s in the train.

Two methods of estimating  $p$  are available:

- 1) The Liley and North equation (1953) estimates  $p$  from the reduction in e.p.p. amplitude of the first two e.p.p.'s. The values thus obtained are plotted against time on a semilogarithmic scale. The value of  $p$  is estimated by extrapolating the curve to zero time (Liley and North, 1953).

- 2) Elmqvist and Quastel (1965) applied similar considerations to a train of end plate responses. In this case, quantal content is plotted against cumulative release, and the size of the readily releasable store

(n) estimated by extrapolating the linear portion of the cumulative regression line to the intercept on the abscissa. The extrapolated intercept on the cumulative axis on an x-y plot is the value of n or the amount of transmitter which would have had to be released for the e.p.p.'s to fall to zero. P is computed as the ratio of the quantal content of the first end plate response to the estimated size of the readily releasable store (n).

Values of p have been estimated to range from 0.14 in frog muscle to 0.25 or 0.45 in d-tubocurarine blocked mammalian muscle (Elmqvist and Quastel, 1965; and Martin, 1966). Readily releasable stores have been estimated using one or the other of these values plus an estimate of quantal content. Usually in these values of n between 300-1000 are found in d-tubocurarine-blocked muscle (Elmqvist and Quastel, 1965). In cut-fiber muscle, however, values of 6000 and 2000 have been obtained (Hubbard, Wilson and Miyamoto, 1969; and Blaber, 1970).

$\text{Ca}^{++}$  ion is of primary importance to quantal release. Gradual reductions in extracellular  $\text{Ca}^{++}$  leads to a step-wise reduction in quantal content of each e.p.p. (Del Castillo and Katz, 1954). Under these conditions, readily releasable stores remain unchanged,

whereas fractional release is markedly decreased (Elmqvist and Quastel, 1965). A similar response can be obtained by increasing  $Mg^{++}$  ion in the Ringer solution. Increasing  $Ca^{++}$  in the Ringer solution results in increased fractional release of transmitter. Thus, quantal content of the first e.p.p. rises. Few drugs appear able to counteract a low  $Ca^{++}$  induced neuromuscular block. Presynaptically it appears that, if this ion is unavailable for mobilization, drugs which increase fractional release by mobilizing  $Ca^{++}$  (e.g. caffeine) (Hofmann, 1969) may possess little activity.

As has been already indicated, there is evidence that entry of  $Ca^{++}$  ions into the nerve terminal controls quantal release. The rate of transmitter release increases very rapidly upon arrival of an action potential at a motor nerve terminal (Katz and Miledi, 1965c). The increased probability of release generated in this way does not immediately return to normal but continues for some time (Rahaminoff, 1968). This is evidenced in 2 ways:

- 1) M.e.p.p. frequency is higher after stimulation (Liley, 1956; Brooks, 1956; Hubbard, 1963; Miledi and Thies, 1967), than under resting conditions.

- 2) Eccles, Katz and Kuffler (1941) and Feng (1940) demonstrated that the second e.p.p. evoked shortly after

the first is of greater amplitude. This phenomenon, called facilitation, declines as the interval between the pair of stimuli is increased.

It has been demonstrated by Del Castillo and Katz (1954) that the facilitation phenomena is due to increased quantal release of ACh. Takeuchi and Takeuchi (1962) suggested that facilitation is due to an increase in amplitude of the second action potential in the nerve terminal. Hubbard and Schmidt (1963), Martin and Pilar (1964), Katz and Miledi (1965c), and Miledi and Slater (1963) have demonstrated facilitation in cases where no increase in the second nerve action potential occurred. Katz and Miledi (1965c) have suggested that facilitation might be due to residual  $\text{Ca}^{++}$  left attached to receptors from the first impulse at the time of arrival of the second impulse. Dodge and Rahaminoff (1967) have postulated that a "co-operative" action of four  $\text{Ca}^{++}$  ions is necessary to release one quanta, while receptor sites containing three or less  $\text{Ca}^{++}$  ions are ineffective. Their theory is based on the principle that dissociation of  $\text{Ca}^{++}$  from all receptors takes several milliseconds. Therefore, on arrival of a second action potential, it would be easier for receptors with partially undissociated  $\text{Ca}^{++}$  to reach the optimum  $\text{Ca}^{++}$  level. Rahaminoff (1968) reported  $\text{Ca}^{++}$



had at least two different effects on facilitation.

1) When the quantal release due to the first impulse was held constant, increased  $\text{Ca}^{++}$  increases the duration of acilitation.

2) When intervals between pulses were shortened, facilitation decreased.

Thus, it would appear that a rather complicated system for transmitter release is present in the motor nerve terminal.

One alternative explanation of facilitation is possible, namely that facilitation resides in one of the unknown subsequent steps which mediate between entry of  $\text{Ca}^{++}$  and transmitter release (Katz and Miledi, 1967). Their results demonstrated that doubling of the pulse duration increased facilitation by more than 50 times. This response was a great deal more than expected on the basis of an exponential decay of  $\text{Ca}^{++}$  dissociation alone.

When  $\text{Ca}^{++}$  ion is kept at a "normal level" and  $\text{Na}^+$  ion reduced, the nerve terminal usually releases a smaller number of quanta per impulse (Liley, 1956). When  $\text{Ca}^{++}$  ion is low, a decrease in  $\text{Na}^+$  produces an increase in quantal content. When  $\text{Ca}^{++}$  is at its normal level, the same decrease tends to produce a small reduction in quantal content. Colomo and Rahaminoff

suggest that reduction in  $\text{Na}^+$  produces two changes. First, more sites are available to binding to  $\text{Ca}^{++}$  (i.e. competition between  $\text{Ca}^{++}$  and  $\text{Na}^+$ ). Second, depolarization of presynaptic nerve terminals is reduced by low  $\text{Na}^+$ , and, therefore, the release mechanism is not fully activated. Similar but more marked results were obtained by Kelly (1965). It is to be pointed out that Colomo and Rahaminoff (1968) indicate that another important function of the reduced e.p.p. amplitude seen in low  $\text{Na}^+$  is due to a reduced postsynaptic sensitivity to ACh.

Birks (1963) demonstrated that lack of  $\text{Na}^+$  has an inhibitory effect on ACh synthesis, and Potter (1968) has demonstrated depressed choline uptake in low  $\text{Na}^+$ .

Kelly (1965) has shown that other monovalent ions can replace  $\text{Na}^+$  ion (e.g. lithium). In certain instances, quantal content was reduced. Therefore, the effect of  $\text{Na}^+$  ion is charge specific.

Katz and Miledi (1966) using tetrodotoxin poisoned muscle selectively prevented entry of  $\text{Na}^+$  into muscle and nerve fibers. They came to the conclusion that release of ACh is evidently not dependent on  $\text{Na}^+$  entry and suggested that the graded relation between the size of the e.p.p. and its quantal content were dependent on the strength and duration of the depolarizing pulse.

They demonstrated that release can be evoked electrotonically and, thus, raised the question of the physiological significance of nerve action potential invasion into nerve terminals.

$\text{Na}^+$  ion can affect transmitter release less directly by depressing the available store of transmitter and/or mobilization rate (Birks and MacIntosh, 1961), or resynthesis of transmitter which is loaded into the available store (Collier and MacIntosh, 1969; Collier, 1969; and Blaber, 1970). The underlying mechanism by which  $\text{Na}^+$  ion affects transmitter stores is, currently, not understood. However, mathematical interpretation of effects on quantal content, readily releasable stores, and probability of release point to mobilization and stores as systems which seem affected.

$\text{K}^+$  ion affects quantal release in a manner opposite from that obtained by applying a depolarizing current (Takeuchi and Takeuchi, 1961; Liley, 1956; and Gage and Quastel, 1965). Parsons, Hofmann and Feigan (1965) demonstrated that  $\text{K}^+$  ion increases the quantal content of each e.p.p. by increasing the probability of release of transmitter and, also, raising the number of quanta in the readily releasable store. The relationship of these data to the increases in m.e.p.p. frequency observed with the  $\text{K}^+$  and depolarization by

applied current to nerve terminals remains a matter of contemporary investigation.

Hofmann (1969) has suggested that caffeine facilitation of quantal release is like that of  $K^+$ . Like  $K^+$ , caffeine can increase the sustained transmitter output. His evidence suggests that the stimulating effect on transmitter release is not limited to surface parameters but involves some metabolic activity within the nerve terminal.

Hofmann, Parsons and Feigen (1966) have found that increased temperature causes a marked increase in quantal release. These authors investigated the effects of veratrine and guanidine as well. Veratrine was without appreciable effect in the absence of  $Ca^{++}$  ion; however, in the presence of  $Ca^{++}$ , veratrine and guanidine had opposite effects. Veratrine blocked transmitter release, while guanidine increased transmitter release. The finding that the rate of sequential depression of e.p.p. amplitudes in a curarized preparation during a train was reduced with increasing temperature and, at the same time, an increase in absolute number of quanta liberated in the initial and succeeding pulses argues in favor of an increase in transmitter stores. It is interesting to note that there occurred a corresponding increase in m.e.p.p.

frequency which could be interpreted as an overflow of production.

The effects of guanidine emphasize certain differences between mammalian and amphibian neuromuscular preparations which are perhaps important to mention here. Otsuka and Endo (1960) observed no changes in m.e.p.p. frequency after addition of guanidine to frog muscle preparations, whereas Hofmann, Parsons and Feigen (1966) found m.e.p.p. frequency to increase markedly. Del Castillo and Engback (1954) have found that m.e.p.p. frequency is independent of external  $\text{Ca}^{++}$  in the frog. Hubbard (1961) has shown that at the mammalian junction there is a significant fraction of m.e.p.p. frequency which is dependent on  $\text{Ca}^{++}$ . It is possible that in mammalian preparations, there is a  $\text{Ca}^{++}$  dependent component of m.e.p.p. release which parallels or is the same mechanism as the  $\text{Ca}^{++}$  dependent release induced by stimulation.

To summarize, it appears that the contemporary literature encompassing the electrophysiological properties of neuromuscular transmission in the mammal has largely centered around identification of a system present in the nerve terminal which controls synthesis storage and release of ACh. The quantal nature of this storage and release system has resulted in the evo-

lution of methods which identify and quantitate several components of the system. Thus, these methods allow estimation of quantal size, quantal content, readily releasable stores, total available store, mobilization, and probability of release.

Experiments designed to identify those factors which control the various components of transmitter storage and release are only beginning. Thus far,  $\text{Ca}^{++}$  appears to be directly involved in the release mechanism.  $\text{Na}^{+}$  seems to serve in a secondary position either controlling the amplitude of the action potential or perhaps, in some way, competing with  $\text{Ca}^{++}$  for entry into the nerve terminal. Another role of  $\text{Na}^{+}$  may be to control mobilization of quanta into releasable locations from a larger less mobile pool (Blaber, 1970).

Correlation of changes in m.e.p.p. frequency (spontaneous release) with quantal release following orthodromic stimulation have shown that considerable similarity exists, thus suggesting that factors which control spontaneous release of quanta also control evoked release. This, however, is not always the case as there are some exceptions (Hubbard, Llina's and Quastel, 1969).

Factors such as the state nerve terminal membrane,

the influence of the action potential time course, and the interval between action potentials on transmitter release are factors only recently being investigated. The relatively new measurable electrophysiological components of transmitter release (e.g. ACh mobilization) have essentially widened the investigational capability of neuromuscular pharmacology. The problem in this field is to identify the site and mechanism of action of a drug.

## PHARMACOLOGY

Neuromuscular pharmacology. - The actions of drugs on neuromuscular transmission stands today as a focus of controversy between several schools of thought. This, at times confusing controversy over the site and mechanism of drug action on neuromuscular transmission, has stimulated considerable research in which new experimental designs have been devised, new drugs synthesized, and novel approaches to therapy of diseases developed. On occasion, studies of drug action on neuromuscular transmission have contributed to revealing certain underlying physiological mechanisms.

Currently, there is no single theory of drug action which explains all the events one can observe during an experiment. For this reason, descriptions of the site and mechanism of drug action have contributed to the development of pharmacology (Clark, 1933) and, perhaps, to its oversimplification (e.g. Standaert and Riker, 1967). One way to express drug action is to identify drug receptors present in the system, then to describe the action of a drug on them.

As far as neuromuscular transmission is concerned, there appears to be an ever increasing number of drug



receptors being identified either at presynaptic sites on the motor nerve terminal or on the postsynaptic sites.

Presynaptic drug receptors were first identified by Masland and Wigton (1940) and Feng and Li (1941) who noted that, in the presence of anticholinesterase agents, the motor nerve responds to a single stimulus with a burst of repetitive antidromic nerve action potentials. The cause of these antidromic potentials remains an unsettled question. Riker (1967), Werner (1960), Barstad (1962), Blaber and Bowman (1963), Randic and Straughan (1964), and Hubbard (1965) have all related antidromic firing to facilitation of neuromuscular transmission, and, further, that facilitation arises in the nerve terminal.

It is presently uncertain whether the antidromic firing obtained, after treatment with anticholinesterase agents, is the result of accumulation of ACh at the neuromuscular junction thus allowing ACh to act at the nerve endings (Eccles, 1964; and Hubbard, 1965), or whether this response is due to a direct action of the particular anticholinesterase drug on the motor nerve terminal. Eccles, Katz and Kuffler (1942) have suggested that antidromic firing may be the result of the prolonged end plate potential arising postsyn-

aptically due to inhibition of junctional cholinesterase. The prolonged end plate current, produced in this way, causes antidromic firing by electrical depolarization of the nerve terminal.

Werner (1960, 1960a and 1961) described two types of antidromic firing occurring as the result of treatment with anticholinesterase agents. One is caused by ephaptic backfiring from repetitive muscle action potentials. The other is due to a prolonged generator potential occurring in the motor nerve terminal.

Blaber and Bowman (1963b) and Hubbard, Schmidt and Yokota (1965) indicated that the prolonged generator potential occurring in the nerve terminal was probably the negative after potential of the nerve terminal spike. This negative after potential appeared to be prolonged by anticholinesterase treatment. Barstad (1962) found that antidromic discharges due to anticholinesterase treatment occur in cut muscle fibers that are unable to produce muscle action potentials. These data support the concept that prolongation of the negative after potential of the invading nerve action potential, if sufficiently intense, could produce repetitive stimulation at the first node of Ranvier and, thus, produce the antidromic discharge (Blaber and Karczmar, 1967).

Identification of drug receptors at presynaptic sites is a complex problem. Much of the information in the literature identifying these sites are method dependent. It is felt by some investigators that there is only one primary site of drug action and this lies on the motor nerve terminal (Standaert and Riker, 1967).

Coers and Woolf (1959) have shown that, in the proximity of the muscle mass, the motor nerve loses its myelin, decreases in diameter, and branches repeatedly. Standaert and Riker (1967) concluded from these data that because of the lack of myelin in nerve terminals, ion, including quaternary ammonium compounds, will have ready access to the nerve membrane and, because of the multiple branching, blockade or facilitation of transmission can easily occur. The small diameter of each branch indicated to them that conduction time would be slower and after potentials longer in the nerve terminal. Unfortunately, direct measurements of the parameters are not readily accomplished in mammalian muscle preparations.

Acetylcholinesterase, the enzyme which hydrolyzes ACh, is present at the muscle end plate and also presynaptically at motor nerve endings (Kolle, 1963). In addition, Dettbarn (1967) demonstrated the presence of cholinesterase at the unmyelinated nodes of Ranvier

on the motor nerve axon. This data supports the concept of Bullock, Nachmansohn, Grundfest and Rothenberg (1947) that anticholinesterase agents may exert their effects on the nerve conduction mechanism. They extended their hypothesis by suggesting that permeability changes that occur in the nerve membrane are controlled by endogenously released ACh (Nachmansohn, 1961, 1962; and Dettbarn and Davis, 1963).

If one considers the concept of Nachmansohn to be true, then inhibition of neuronal cholinesterase could lead to prolonged depolarization of the membrane by prolonging the ACh permeability change where cholinesterase is present. This increased permeability could be a factor contributing to antidromic firing, in that electrical threshold of the nerve membrane may be altered. It is also possible that if the permeability change is prolonged at the nodes of Ranvier, conduction block could also ensue.

Ritchie (1967) has reviewed his studies on mammalian non-myelinated nerve fibers (Armett and Ritchie, 1960, 1961, 1962, 1963; and Ritchie, 1965). In these experiments in which the sucrose gap technique was utilized, ACh produced a rapid depolarization of the neuronal membrane. The action potential was depressed, the positive after potential prolonged, and conduction

slowed. At high concentrations conduction block was obtained. The action of ACh on nerve conduction appears to be nicotinic in nature, in that equimolar amounts of carbachol and nicotine produce the same effect on nerve conduction, whereas muscarinic drugs were inactive (Armett and Ritchie, 1961). Analysis of experiments, in which  $\text{Na}^+$  and  $\text{Ca}^{++}$  were altered extracellularly, indicated that a portion of the ACh induced depolarization may be the result of increased permeability to  $\text{Ca}^{++}$  ions.

D-tubocurarine was demonstrated to block the effect of externally applied ACh in these nerve fibers (Armett and Ritchie, 1961). In addition to antagonizing ACh, high concentrations of d-tubocurarine alone can produce conduction block (Armett and Ritchie, 1963). However, this effect differs significantly from that seen postsynaptically at the muscle end plate, as the observed conduction block is accompanied by neuronal depolarization and this does not occur at the end plate. Thus, this portion of Ritchie's work suggests that there are indeed nicotinic receptors present on motor axons.

Other studies (Ritchie, 1967) argue against a physiological role of ACh in nerve conduction in that neostigmine did not alter nerve action potential con-

duction or affect the resting potential. Physostigmine, on the other hand, blocks conduction without depolarizing the fibers. Thus, two reversible cholinesterase inhibitors have different actions at the nerve membrane. There is doubt, therefore, concerning the role of the acetylcholinesterase that is associated with a neuronal membrane. It would not appear justified to assume that inhibition of axonal cholinesterase would lead to depolarization blockade of action potential conduction via increased endogenous ACh.

In addition to the identification of cholinergic receptors present in the motor nerve axon, as demonstrated by Ritchie, and in the nerve terminal, as evidenced by the presence of antidromic firing in nerve muscle preparations treated with anticholinesterase agents, a number of reports in the literature have indicated a motor nerve terminal action of succinylcholine (SCh) and decamethonium ( $C_{10}$ ) (Edwards and Ikeda, 1962; Segawa, Kojima and Takagi, 1965; Standaert and Adams, 1965; Wikinski, Usubiaga, Usubiaga and Wikinski, 1965; and Blaber and Karczmar, 1967). The succinylcholine and decamethonium induced antidromic discharges resemble those observed with anticholinesterase treatment and are readily blocked by d-tubocurarine.

Development of the intracellular microelectrode method for the electrophysiological study of synaptic transmission introduced a new approach and provided different information on the effects of drugs on neuromuscular transmission.

Although early studies of neuromuscular transmission assigned the postsynaptic end plate region as the site of the nicotinic receptor (Brown, Dale and Feldberg, 1936; and Eccles, Katz and Kuffler, 1941, 1942), direct intracellular recordings of postsynaptic phenomena began to take on pharmacological significance with the work of Fatt and Katz (1950, 1952). In these studies they found that the amplitude and half decay time of m.e.p.p.'s were significantly increased by treatment with neostigmine. If the m.e.p.p. was the electrical result of a combination of a single quanta of ACh with its nicotinic receptor, then preventing the hydrolysis of ACh should prolong the time course of this receptor interaction and consequently alter the time course of the electrical event. Complementary to these studies of m.e.p.p.'s were the earlier studies in which the e.p.p. was recorded extracellularly (Jenkinson, 1960). These data indicated that d-tubocurarine decreased the amplitude of the e.p.p. by competitive antagonism at the cholinergic receptor.

Subsequent treatment of a curarized preparation with anticholinesterase agents results in an increase in the amplitude and duration of the e.p.p. (Feng, 1940; Eccles, Katz and Kuffler, 1942; and Eccles and MacFarlane, 1949).

In addition, it was noted that when the e.p.p. was sufficiently prolonged, repetitive firing of the muscle membrane occurred.

When it became apparent that a facilitatory drug theoretically could exert its action by both pre and postsynaptic mechanisms, numerous studies were undertaken to correlate the cholinesterase inhibiting potency of a compound with its facilitatory action on muscle. These studies, although extensive, can only be summarized as equivocal. Several schools of opinion arose from this controversy. The actual degree by which anticholinesterase agents facilitate neuromuscular transmission by inhibiting cholinesterase is unknown as yet. Standaert and Riker (1967) categorically refuse to accept this hypothesis. Blaber (1960, 1963) and Blaber and Bowman (1959) were unable to correlate anticholinesterase potency with d-tubocurarine reversal or twitch increase due to treatment with neostigmine or edrophonium.

During this period of controversy, Karczmar (1967)



and Blaber and Karczmar (1967) proposed their theories of multiple cholinceptive and related sites at the neuromuscular junction. They proposed five cholinceptive sites at the neuromuscular junction of the cat: the motor end plate, acetylcholinesterase, the motor nerve terminal, producing antidromic discharges in the motor nerve following orthodromic stimulation, the motor nerve terminal producing depolarization of the first node, and the choline carrier mechanism. The choline carrier mechanism was added to typify the novel neuromuscular paralyzing action of the hemicholiniums. These agents appear to depress neuromuscular transmission by preventing uptake of choline (a precursor to ACh synthesis) by the nerve terminal (Angeles, Schueler, Lim and Sotto, 1964; and Evans and Wilson, 1964).

"Multiple sites of drug action" seems to offer a rational explanation of the evidence currently at hand. It appears that this evidence does indeed point to nicotinic receptors on the nerve axon, nerve terminal, and postsynaptically at the end plate region. The predominance of one receptor over another may depend on the conditions of the experiment, the species of animal, the type of tissue, the type of drug, and most importantly the dose selected (Blaber and Karczmar,

1967).

With the discovery of the quantal nature of transmitter release by the intracellular microelectrode methods (Del Castillo and Katz, 1954), additional data began to appear in the literature on the effects of drugs on ACh quanta.

In studying m.e.p.p.'s in isolated cat tenuissimus muscle, Boyd and Martin (1956b) found prostigmine to increase their amplitude and time course. This effect paralleled a corresponding increase in m.e.p.p. frequency. Thus, in this case, they identified a pre and postsynaptic site of action.

Edwards and Ikeda (1962) reported on the effects of 2-PAM and succinylcholine on quantal release. Their studies showed the quantal content of the e.p.p. decreased with succinylcholine while 2-PAM, a cholinesterase reactivator, increased quantal content. They considered the effects of 2-PAM consistent with its anticholinergic action and the effects of SCh consistent with its blocking action.

Christ (1969) studied the effects of a variety of facilitatory drugs on m.e.p.p.'s, e.p.p.'s, quantal content, and iontophoretic response to ACh. Neostigmine caused a significant increase in m.e.p.p. frequency. At increasing doses, amplitude and time course of m.

e.p.p.'s increased, at still higher doses the muscle membrane depolarized. E.p.p.'s were found to increase with "no change in time course", unless the dose was again increased. Edrophonium, another "cholinesterase inhibitor" produced a marked increase in m.e.p.p. frequency. Concentrations of 1000x that which increased m.e.p.p. frequency were without effect on m.e.p.p. time course. E.p.p.'s on the other hand were increased and prolonged by edrophonium.

In contrast to these data, no change in m.e.p.p. frequency was observed in frog sartorius muscle treated with neostigmine (Fatt and Katz, 1952) or in rat diaphragm (Liley, 1956).

Studies with iontophoretic potentials demonstrated that cholinesterase inhibitors prolonged time course thus suggesting a postsynaptic action for these compounds.

In 1969, Hofmann investigated the effects of caffeine on transmitter depletion and mobilization at motor nerve terminals. His data based on an analysis of trains of e.p.p.'s suggested caffeine may have an effect on transmitter mobilization in the nerve terminal.

Beranek and Vyskocil (1967) reported that d-tubocurarine reduces the depolarizing action of ion-

tophoretic ACh potentials much less than it reduces the amplitude of the e.p.p. Hubbard, Wilson and Miyamoto (1969) investigated the effects of d-tubocurarine on several parameters of transmitter release. They found a marked reduction in quantal size which is consistent with the postsynaptic effects of this agent. D-tubocurarine also increased probability of release, decreased quantal content, markedly depressed readily releasable stores, and depressed mobilization. The authors suggested that in addition to its well known postsynaptic effects, there occurred long acting presynaptic effects of d-tubocurarine that may be due to an intracellular action on the nerve terminal.

Blaber (1970) has studied the facilitatory action of  $C_{10}$  on the same parameters of transmitter release as Hubbard. His studies demonstrated that, at low doses,  $C_{10}$  increases the quantal content of the e.p.p. by increasing mobilization and consequently the readily releasable store. He concluded that there is a cholinergic site on the unmyelinated nerve terminal that, under certain circumstances, can be facilitatory.

Still other drug receptors may be present in the neuromuscular junction that are of a non-cholinergic nature.

Oliver and Schafer (1895) demonstrated that ex-

tracts of the adrenal medulla increased muscular tension and contraction time in dog gastronemus muscle. Epinephrine was subsequently shown to possess an anti-curare action (Rosenbleuth, Lindsley and Morison, 1936; Wilson and Wright, 1937; Maddock, Rankin and Youmans, 1948; and Naess and Sirnes, 1953). Bulbring and Burn (1942), Naess and Sirnes (1954), and Blaber and Bowman (1963) found epinephrine to augment the repetitive firing and the twitch potentiation produced by anticholinesterase drugs. Paton and Zaimis (1949) found similar effects in the presence of depolarizing blocking drugs ( $C_{10}$  or SCh).

Bowman and Raper (1967) found that all the direct actions of sympathomimetic amines on muscle were unaffected by dibenamine, an alpha receptor blocking drug. However, they were abolished by dichloroisoproterenol, a beta receptor blocking drug. From these data and the finding that 1-isoproterenol was more potent than norepinephrine, the possibility arose that there was a beta receptor present in skeletal muscle fibers. On the basis of studies in denervated muscle, fast contracting muscle, and slow contracting muscles (Bowman and Raper, 1967), it was concluded that activation of beta receptors increases muscle tension in both fast or slow denervated muscle. An interesting

contrast arises in normal muscle. In this case, activation of beta receptors facilitates neuromuscular transmission in fast muscle and depresses neuromuscular transmission in slow muscle.

Cross-innervation of a slow muscle with motoneurones, formerly innervating a fast muscle, converts the slow muscle to a fast muscle (Buller, Eccles and Eccles, 1960). Bowman and Raper (1962) found a corresponding reversal of the pharmacological properties of epinephrine in cross-innervated muscle. In this case beta receptor activation facilitated neuromuscular transmission in the slow muscle which had been cross-innervated with motoneurones from a fast muscle.

The presence of an alpha receptor in the neuromuscular junction is based on the investigations of Maddock, Rankin and Youmans (1948) in which they demonstrated that the anticholinergic action of epinephrine could be prevented with prior treatment by dibenamine (an alpha receptor blocker). These facilitatory effects of epinephrine are produced equally in both fast and slow conducting muscles. Although epinephrine and norepinephrine produce an anticholinergic effect, isoproterenol possesses little activity.

In addition to an anticholinergic action, epinephrine and norepinephrine have been shown to potentiate the

twitch facilitation produced by neostigmine and decamethonium (Bowman and Raper, 1967). Repetitive firing produced in motor nerve terminals by anticholinesterase drugs are also augmented by epinephrine (Blaber and Bowman, 1963). All of the alpha effects produced by epinephrine are unaffected by beta receptor blocking drugs but are inhibited by the alpha receptor blocking drugs (Bowman and Raper, 1967).

Evidence that alpha receptors, which can be activated by epinephrine or norepinephrine, are present at presynaptic sites on the motor nerve terminal is suggested by that data of Krnjevic and Miledi (1958). They found that epinephrine could relieve presynaptic failure in rapidly stimulated nerve-muscle preparations and augment trains of e.p.p.'s. Ionophoretic potentials produced by ACh were not affected. Bowman and Raper (1966) suggest that the alpha receptor activation leads to hyperpolarization of motor nerve terminals.

Still another drug receptor site that is present in skeletal muscle is evidenced by the facilitatory actions of the methyl xanthines. These drugs (e.g. theophylline) are known to inhibit the enzyme phosphodiesterase and subsequently prevent the breakdown of cyclic 3'5'AMP to adenosine-5-phosphate.

Breckenridge, Burn and Matschinsky (1967) found

that theophylline potentiated muscle strength (tension) increases induced by prior administration of epinephrine or neostigmine. From their experiments carried out using cat gastrocnemius muscle in situ, they found that theophylline was without effect on directly stimulated muscle. Breckenridge, Burn and Matschinsky (1967) proposed a role for cyclic AMP in augmenting the release of ACh.

Fluoride on neuromuscular transmission. - Our decision to examine the effects of sodium fluoride (NaF) were related to several contemporary problems in neuromuscular pharmacology. Of these, two objectives are clearly relevant to this thesis. The first of these relates to the unusual pharmacological properties of this ion, and the second to the possibility of identifying additional drug receptors which might be present at the neuromuscular junction.

Contemporary work with fluoride was initiated by Koketsu and Gerard in 1956. These investigators identified an anticurare action of fluoride that could not be attributed to an anticholinesterase action of this compound. Koketsu (1965) proposed that the anticurare action of fluoride was due to a sensitizing action of fluoride on the end plate region of the muscle,



thus suggesting a new mechanism of reversing a d-tubocurarine blockade. His evidence for this concept of a d-tubocurarine antagonism centers around the results of intracellular microelectrode studies in frog sartorius muscle. The results of Koketsu can be divided into five categories: 1) effects on m.e.p.p.'s; 2) effects of e.p.p.'s; 3) sensitivity of the end plate to exogenous ACh; 4) effect on membrane potential and electrical threshold; and 5) effect on mechanical response.

The effect of NaF on m.e.p.p.'s recorded intracellularly appeared in his studies to be due largely to postsynaptic effects. In this case, m.e.p.p. amplitude substantially increased over a 20 minute treatment period, while concentrations of 0.5 to 10mM NaF showed no effect on m.e.p.p. frequency. Concentrations in the range of 3mM NaF increased m.e.p.p. amplitude by 3 fold. At times, m.e.p.p. amplitude increased to the point where muscle action potentials could be elicited.

In studying e.p.p.'s, Koketsu utilized the methods of Fatt and Katz (1951). In this case, an isolated sartorius muscle was blocked by d-tubocurarine, and e.p.p.'s located by intracellular microelectrode technique. NaF at 0.5mM to 10mM decurarized the paralyzed muscle. During the course of NaF treatment, the re-

corded e.p.p.'s progressively increased in amplitude. A maximum increase in amplitude of the e.p.p. was correlated with appearance of action potentials on the peak of the e.p.p.

An unusual end plate response was obtained in preparations blocked by severe tetanization. Perfusion of 2mM NaF increased these e.p.p.'s nearly 3 fold. In this case, however, muscle action potentials appeared on the declining phase of the e.p.p. Koketsu suggested that the muscle action potential appearing on the declining phase was made possible by a reduction of the threshold of the muscle fiber close to the end plate region.

In another series of experiments, e.p.p. half fall was increased only slightly by NaF. Koketsu did not attribute this action of fluoride to the anticholinergic action of the fluoride, since augmentation of the peak potential and changes in threshold were more markedly changed in the periods preceding restoration of the muscle action potential than were changes in e.p.p. time course.

The sensitivity of the end plate region was studied by measuring surface potential changes produced by ACh in the presence and absence of NaF. In these experiments, the distribution of surface poten-

tial over a muscle mass is detected by changing the level of an ACh containing Ringer's solution every minute for 2-30 minutes. Regions of muscle containing end plates were identified by peak depolarization recordings obtained at certain fluid levels. These measurements demonstrated that NaF in concentrations of 0.1 to 10mM increased the peak depolarization obtained without altering its time course. Eserine, on the other hand, increased and prolonged the time course of the peak depolarizations. These data supported the concept of Koketsu that the mechanism action of NaF involved some other site than acetylcholinesterase.

Mechanical responses in frog sartorius muscle were measured by Koketsu under three different conditions. Effects of fluoride were assessed in the absence of any neuromuscular block, in the presence of d-tubocurarine block, and in muscle partially blocked by severe tetanization.

In the absence of neuromuscular block, NaF up to 10mM failed to produce any augmentation of muscle twtich, however, fibrillations were noted. Preparations blocked by d-tubocurarine were readily reversed by NaF. When strong repeated tetanization was applied to produce partial neuromuscular block, muscle tension was also restored by NaF.

From these studies, Koketsu concluded that fluoride did not antagonize d-tubocurarine by increasing the release of ACh from presynaptic sites, nor did the decurarization obtained correlate with the effects of anticholinesterase agents on neuromuscular transmission. He concluded that NaF augments the e.p.p. and m.e.p.p. amplitude by increasing the sensitivity of the end plate to ACh. Sensitivity was explained by Koketsu as an increased affinity of ACh for the end plate receptors coupled with a reduction in electrical threshold of the muscle membrane close to the end plate region.

In 1965 Koketsu reported that NaF appeared to possess a restorative action in frog preparations in which synaptic transmission had been blocked by organophosphorous anticholinesterases. This unexpected new action of fluoride was independently studied by Heilbronn (1964) using in vitro biochemical methods. Although the novelty of this action on isolated preparations was clear to both authors, each came to separate conclusions as the mechanism of fluoride action.

Heilbronn (1964, 1965) and Albanus, Heilbronn and Sundwall (1965) have presented in vivo and in vitro data which indicated that NaF acts as a reactivator of acetylcholinesterase which had been inhibited "irreversibly" by organophosphorous anticholinesterases.

Koketsu (1966) and Karczmar, Koketsu and Soeda (1968) have indicated that the restoration of transmission obtained with fluoride appears to be due to a dual action, namely 1) reactivation of phosphorylated cholinesterase and 2) the resensitization of the postsynaptic membrane to ACh. Their data supporting resensitization is based on evidence showing a brief potentiation of the falling phase of the e.p.p. by NaF. The enhanced failing phase occurred during an organophosphorous induced block. Presumably, the sensitizing action of fluoride removes the desensitization of the end plate membrane caused by a sustained action of the accumulated ACh, and, consequently, this action causes a temporary potentiation of the falling phase of the e.p.p.

Albanus, Heilbronn and Sundwall (1965) were unable to explain reversal of neuromuscular block on the basis of reactivation of cholinesterase alone, because the reversal obtained occurred more rapidly than would be expected.

Blaber (1970) studied the effect of various concentrations of NaF on the isolated rat diaphragm in the presence and absence of an organophosphorous induced neuromuscular blockade. 2mM NaF, tested in the absence of anticholinesterase blockade, was observed to pre-

vent the deterioration of twitch and tetanus height commonly seen with this preparation over a 3 hour test period. Post tetanic potentiation was depressed by fluoride. Similar effects of fluoride were seen at 4mM. At a concentration of 8mM NaF both twitch and tetanus height were depressed after about 1 1/2 hours of continuous exposure. Post tetanic potentiation was depressed to a greater degree following 8mM NaF than seen after 4 and 2mM NaF.

In the presence of organophosphorous agents, NaF restored neuromuscular transmission in a manner similar to that seen with N-Methylpyridinium-2-Aldoxime Methane Sulphonate ( $P_2S$ ).

Karczmar, Koketsu and Soeda (1968) compared various sensitizing agents for their effects on the e.p.p. amplitude. They found that d-tubocurarine, as well as P-2-AM, had a reactivating action. Further, P-2-AM resembled NaF in that the former compound also increased ACh depolarization and e.p.p. amplitude without affecting the duration of either. These authors suggested that oximes, methoxyambenonium, and NaF belong to a group of compounds which seem to combine sensitizing or facilitating actions with reactivator effects.

Interesting data in frog rectus abdominus muscle was obtained by Vallette and Ozan (1964). In their

studies, it was found that 4mM NaF profoundly augmented the muscle contracture to KCl. Similar data were obtained using guinea pig ileum. Matthews (1970) suggested that this action may be due to an influence of NaF on membrane excitability.

Caruso, Maynard and DiStefano (1970) studied the effect of NaF on dog respiratory muscle and isolated gastrocnemius muscle in situ. After an i.v. injection of 40mg/Kg NaF respiratory rate increased as did force of contraction of the gastrocnemius muscle. After 32 minutes muscle contraction became depressed in the presence of an elevated respiratory rate. In other experiments these investigators found that NaF reversed a d-tubocurarine blockade but not the neuromuscular blockade produced by decamethonium, a known depolarizing agent. These latter data are in contrast to the findings of Koketsu and Karczmar (1966) who reported that NaF does indeed antagonize a decamethonium blockade.

It became apparent that NaF may represent an interesting drug to investigate for its effect on mammalian fast twitch muscle, particularly in view of the potential differences in drug response obtained in various species, and the need to identify drug receptor systems that may predominate. Our objective

was to investigate specifically the anticurare action of NaF in cat tenuissimus muscle and determine what mechanism or mechanisms contribute to restoration of normal muscle tension.

To accomplish these ends I approached the problem from two aspects: 1) what was the action of NaF on normal tenuissimus muscle? and 2) what was the action of NaF on d-tubocurarine paralyzed tenuissimus muscle? The methods used center primarily around unicellular micro-electrode procedures. In this thesis I present the effects of fluoride on muscle tension, resting membrane potential, miniature end plate potential amplitude and frequency, quantal size, quantal content, probability of release, and readily releasable stores of neurotransmitter and mobilization. Studies on the time course of m.e.p.p.'s and e.p.p.'s are also presented. All of these components of neuromuscular transmission were examined both in the presence and absence of d-tubocurarine.



## METHODS

Muscle contraction experiments. - Dale and Gaddum (1930) described experiments on the denervated kitten diaphragm suspended in a bath. Bülbring (1946) adopted this procedure for pharmacological studies in the albino rat.

In vitro nerve muscle preparations historically were evolved to study physiological as well as pharmacological problems in nerve muscle transmission primarily to avoid compensatory and feed back mechanisms present in vivo. For example, vascular changes occurring in vivo can alter twitch responses to injection of ACh or adrenaline (Bülbring and Burn, 1940).

The in vitro method is however not without problems. Factors such as adequate oxygenation, temperature, pH, and removal of waste products are factors which must be taken into account in any construction of a test system. These factors must be also considered in the interpretation of results obtained.

In practice, it must be emphasized that much of the methodology historically has been developed by trial and error. Investigators often made adjustments in their system to improve tissue responses or to maintain them. Most physiological salt solutions are mod-

ified from the salt solution of Ringer (1894). The most commonly used are Locke-Ringer (1901), Tyrode (1910), Krebs (1950), and Krebs-Henseleit (1932) solutions. These salt solutions compare favorably in ionic composition with either human, rat, or frog extracellular fluid (plasma) Krebs, 1950). Numerous modifications of the above solutions have been made for each type of tissue studied. For example, Bülbbring's solutions is a modification of Tyrode's solution with 2x normal glucose. McEwan's solution constitutes still another modification of Tyrode's solution.

Arbitrary alteration or selection of a physiological salt solution is not wise in view of the wide discrepancies already apparent in the literature. Such measurements as oxygen uptake by tissue varies considerably from species to species and are subject to marked alteration by adjusting ionic composition of an artificial medium (Krebs, 1950).

Since serum or plasma is the natural environment of animal tissues, one may consider its composition the most suitable to mimic. Plasma requires the addition of anticoagulants (i.e. NaF), which, obviously, makes it unsuitable for study. In the intact animal, the balance of activities of all organs maintain a relative constancy of the concentration of serum

constituents, but in vitro metabolic activity may rapidly convert serum into a less physiological salt solution (Krebs, 1950).

Another factor to be taken into consideration is diffusion. In vivo, the diffusion path is shorter than in vitro. The average distance between capillary wall and tissue cell is much shorter than the distance between the surface and the center of a muscle mass isolated in vitro.

Difficulties due to rapid exhaustion and slow diffusion have been overcome by increasing the concentration of the 'relevant' metabolites in the medium.

Krebs (1950) has attempted to identify the 'relevant' constituents in tissue respiration. He suggested that serum be supplemented by adding the following isotonic substrate solutions per 100 parts serum: 3 parts of 0.16 sodium pyruvate, 6 parts of 0.1M sodium fumarate, 3 parts of 0.16M sodium glutamate, 5 parts of 0.3M glucose. The solution should be in equilibrium with a gas mixture of 5% CO<sub>2</sub>.

Since serum contains unknown and variable constituents which are difficult to obtain, and may contain inhibitory antibodies, it is considered advantageous to use a serum substitute. The earlier serum substitutes (e.g. Ringer's solution) were prepared

on an empirical basis. Ringer (1882, 1883, 1886) tested various saline preparations on the isolated frog heart and found certain concentrations of  $\text{Ca}^{++}$ ,  $\text{Na}^+$ , and  $\text{K}^+$  were required for maintenance of a normal beat. Locke (1901), Barkan, Broemser and Hahn (1921), Warburg and Uyesugi (1924), and Krebs and Henseleit (1932) composed their serum substitutes from analysis of serum composition of inorganic constituents and glucose.

In vitro isolation of a muscle preparation significantly alters oxygen uptake at the cellular level. Barcroft and Dixon (1907) and Meyerhof and Shulz (1929) have shown that the state of activity of the muscle may cause as much as a thirty-fold increase in resting rate of respiration. Krebs and Henseleit's artificial salt solution contains the following composition in meq/L:

sodium 143.5meq/L	phosphate morobasic 1.2meq/L
potassium 5.4meq/L	bicarbonate 24.9meq/L
calcium 5.1meq/L	sulfate 2.4meq/L
magnesium 2.4meq/L	glucose 10meq/L
chloride 128.0meq/L	

This solution is maybe less physiological than Kreb's (1950) which contains 21meq of organic anions.

Bülbring selected Tyrode's solution originally for use with the isolated rat diaphragm preparation (1946). Recent studies and the experiments in this dissertation were carried out using the solution of

Krebs-Henseleit (1932).

A female albino rat (150-250gm) is killed by a blow on the head. The animal is positioned on its back, the fur overlying the thorax stripped away, and the muscles freed from the chest wall and cut away. A small hole is made on either side of the rib cage cephalic to the marginal region of the diaphragm. This hole is enlarged by cutting through the ribs alongside the base of the sternum and, after visualizing the position of the left or right phrenic nerve, the ribs are cut through towards the animal's flank. The marginal rib continuous with the diaphragm is left intact. The upper part of the thorax is removed completely, and the phrenic nerve traced up to the region of the thymus where a ligature is placed. The phrenic nerve is sectioned above the ligature, then carefully teased free and trimmed of some of the attached connective tissue to a point several millimeters above its entry into the diaphragm muscle mass. An incision is made in the abdominal wall below the diaphragm freeing it from the abdominal cavity. Two incisions are then made towards the vena cava transecting the diaphragm, one lateral to the sternum and the other lateral to the spine. These cuts are made in such a manner that a fan-shaped segment of diaphragm muscle is pro-

duced. The roots of the phrenic nerve are centrally located in the wedge about 2-4mm from the apex. A long thread is attached to the apex of the diaphragm section for recording muscle movement. The marginal edge of the diaphragm (wide base of the wedge) and its associated rib are fastened securely to a perspex rod. The phrenic nerve is carefully laid over bipolar platinum electrodes attached to the perspex muscle holder in a manner which prevents the muscle fibers from making contact with the electrodes. The apex of the muscle wedge stands vertically and the long attached thread is connected from this point directly to the lever arm of a Phipps and Bird linear force transducer. The lever is held in a horizontal position by a light-weight coil spring. With this system a muscle twitch pulls downward on the lever. The movement produced is recorded using a suitable amplifier and recorder. This transducer arrangement provides a reasonable friction free isotonic response to occur. Conversion of the muscle to electrical response is linear over a range of several millimeters of muscle movements as long as the lever arm and associated wire loops move over a central portion of the induction coil.

The entire nerve-muscle preparation, muscle holder, and attached electrode are suspended beneath the trans-

ducer in a 50ml double wall tissue bath (Metro Corp.).

Krebs-Henseleit solution is brought to 37°C by prewarming in an Anderson coil. The isolated tissue is bathed by gravity feed of 50ml of the salt solution into the tissue bath. The temperature of the bathing solution is maintained by pumping the warming solution through the outer chamber of the bath.

Oxygen containing 5% CO<sub>2</sub> is forced, under 2-3 lbs per square inch of pressure, through a sintered glass filter in the base of the tissue bath thus bringing the Krebs-Henseleit solution into equilibrium with the gas.

Recording of muscle movement is accomplished with a Texas Instrument Co. oscillo/riter.

Contractions were elicited by indirect stimulation of the phrenic nerve stump. The bipolar electrodes are connected to a Grass model S-8 square wave stimulator. Pulses at a selected frequency were delivered at 2x the voltage required to elicit a maximum contraction. Pulse duration did not exceed 0.2msec.

Three basic experimental procedures were used to assess the qualitative features of the anticurare action of NaF. Three muscle specimens were selected, rat diaphragm, kitten diaphragm, and isolated cat tenuissimus muscle. In all three experiments the procedures

were identical. Recording system, bathing fluid, pH, and bath temperature were the same.

In the case of kitten diaphragm and cat tenuissimus muscle, the animals were anesthetized with 60 mg/Kg of alph-chloralose (City Chemical Corp.) and 6.0mg/Kg pentobarbital sodium (Abbott Lab.) administered intraperitoneally. 1.5mg/Kg atropine sulfate (Merck and Co.) was included in the injection to minimize effects of excessive parasympathetic stimulation produced by the alph-chloralose.

The protocol used in all three preparations reported was of a qualitative nature. I needed to know what concentration range of NaF functionally antagonized a d-tubocurarine block. In addition, I wished to know the effects of NaF on muscle movement in the absence of d-tubocurarine. D-tubocurarine was supplied by Abbott Laboratories and NaF by Fisher Scientific Co. Injection volumes of all drugs were restricted to 0.1-0.5ml (less than 1% of bath volume). Adjustments for osmotic strength changes due to drug administration were not made.

Intracellular microelectrode methods. - Male or female cats, usually 2-3Kg in body weight, were anesthetized with a mixture of alpha-chloralose, pento-



barbital sodium, and atropine in the same manner and using the same doses as previously described for muscle movement experiments.

The tenuissimus muscle, together with a section of its nerve, was removed from the anesthetized animal by the following technique:

A hind limb was shaved over the popliteal space, and the overlying skin was stripped away. After cauterizing blood vessels to the skin and popliteal fat pads, the tenuissimus muscle becomes easily visible as a long thin strip of superficial muscle coursing along the surface of the underlying muscle. The tenuissimus is freed from its connective tissue and ligatures placed at proximal and distal positions, so that the points where the bifurcated nerve enters the muscle lies approximately in the center between the two ligatures. The overall length of muscle used is about 5.0cm. The tenuissimus nerve is traced upwards towards the point where it becomes continuous with the sciatic nerve trunk. For a distance of several millimeters, the nerve courses along the surface of the sciatic before it can no longer be visualized. Careful clearing of connective tissue allows separation of 3-5cm of nerve.

The tenuissimus nerve muscle preparation is mounted in a constant temperature bath and irrigated with

Krebs-Henseleit solution preheated to  $37 \pm 1^\circ\text{C}$  and oxygenated with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$  gas. (fig. 1).

KCl microelectrodes of 5-15m resistance were prepared using a vertical glass electrode puller (Kopf Fast. Co.). The techniques employed for recording with glass capillary microelectrodes have been described by Fatt and Katz (1951). This microelectrode method was initially developed by Graham and Gerard (1946), Ling and Gerard (1949), and Nastuck and Hodgkin (1950). In the experiments reported here, electrical communication to recording devices was accomplished by means of a silver-AgCl coated platinum wire inserted in one end of KCl filled microelectrode. The leads from the two electrodes are connected to a battery operated neutralized input capacity amplifier (Bioelectric model NF1). This amplifier has a "neutralized" gain of 2-5x. A 100mV square wave fed through a resistance of 22 megohm will produce a wave form with a maximum rise time of 5sec after neutralization. Overshoots of the waveform, slanting, and other undesirable characteristics are visibly corrected with neutralization. Power to the amplifier is by 12 mercury cell batteries.

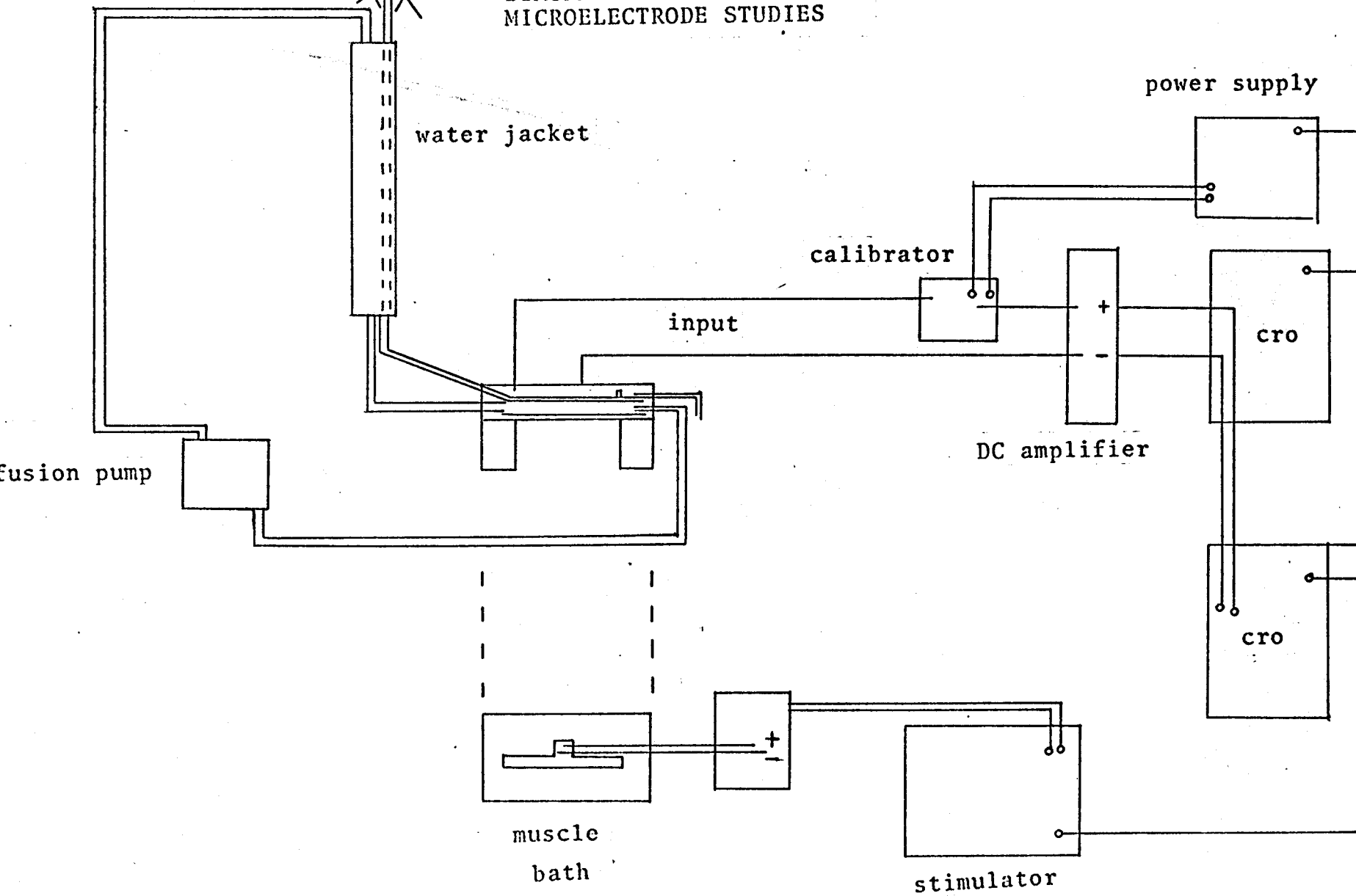
Calibration pulses were produced by introduction into the recording circuit of a bioelectric CA-5 calibrator. The circuit is arranged, so that the signal

O<sub>2</sub>

resevoir

FIGURE 1

DIAGRAM FOR INTRACELLULAR  
MICROELECTRODE STUDIES



recording instruments and amplifier process the calibration pulse and the electrical activity of the biological preparation in an identical manner.

Two types of oscilloscopes were used in displaying tissue electrical activity. A dual beam type 564 Tektronix storage oscilloscope was used for monitoring purposes. Desired data for recording purposes were displayed on a type 502 dual beam Tektronix oscilloscope and photographed with a Nihon-Kohden continuous recording 35 mm reflex type motion picture camera.

Thus, for a desired experimental procedure, single frames displaying calibration pulse and intracellular biological electrical events could be recorded permanently on film. The continuous motion mode allows spreading out of rapidly occurring events. Electrical responses to be measured were enlarged with a Durst 606 35mm enlarger and displayed on mm graph paper. Units of electrical responses (e.g. millivolts) were derived by determining the enlarged amplitude of a suitable calibration introduced periodically during the course of each experiment and converting each mm response into millivolt equivalents.

The irrigation chamber was constructed of perspex and consisted of a chamber about 0.8cm wide and 7cm

long. The base of the chamber was constructed of thicknesses of cork built up to provide an overall depth of 0.5cm. A dam at one end of the chamber controlled bath depth to about 0.3cm. As solution perfused the chamber, it coursed along in the direction of the overflow dam. A reservoir of Ringer was maintained at a constant level with a venopak constant drip apparatus.

The level of fluid in the reservoir is 21 inches above the level of the muscle chamber and, thus, provides gravity feed. The reservoir is connected to the muscle chamber, first through a section of rubber tubing, then a length of polyethylene tubing (PE 160:I.D.= 0.0145") which coils through a warming chamber constructed from a 100ml plastic graduate cylinder. The tubing exits the warming chamber approximately four inches above the level of the bath and connects with the muscle chamber through a small hole in the wall. Temperature is maintained in the bath by circulation of warm water in a false chamber surrounding the base of the muscle chamber. The volume of the muscle chamber is about 3ml ( $2.8\text{cm}^3$  by calculation). The combination of the height of the reservoir column and diameter and length of the connecting tubing determine the rate of flow of fluid through the bath. Since the tubing was periodically replaced, variations in perfusion rate

were evident. In general, perfusion through the bath occurred at a rate of 5-10ml per minute. This rate range allowed acceptable exchange of the bath volume. Within experiments perfusion rate remained constant. The depth of fluid above the muscle surface was about 3mm. This varied with the size of the muscles.

The electrodes for nerve stimulation were situated in an adjacent chamber separated from the muscle bath by a thin perspex shield with a small hole drilled in it. There was sufficient leakage in the chamber to allow constant exchange of perfusion fluid from the main muscle chamber. Oxygenation was accomplished by bubbling 95% O<sub>2</sub>, 5% CO<sub>2</sub> into the reservoir.

The entire chamber rests on a microscope base fitted with a convex mirror and light in such manner that the central portion of the muscle chamber is indirectly illuminated. When the muscle is pinned in position, the focused beam of light allows detailed visualization of a portion of the muscle surface through use of a Leitz dissecting microscope.

Positioning of the microelectrode is accomplished through use of a Narishige micromanipulator.

In practice each experiment followed a protocol which was convenient and reasonably efficient. Each cat used was fasted overnight prior to anesthesia.

This procedure minimized hazards that may be occasioned by vomiting after administration of anesthetic and reduced defecation and urination during the operative procedure.

The anesthetic was administered intraperitoneally. General anesthesia occurred some 30-40 minutes later. During this period, the Krebs-Henseleit solution was prepared, and the heating pump supplying warm water to the warming chambers was turned on. Oscilloscopes were switched on as well.

Perfusion was accomplished through two identical reservoirs - one for control perfusion and one for drug treatment which are connected to a common polyethylene tube via a T-connector. Cross contamination of the reservoirs was avoided by use of appropriate pinch clamps. The reservoirs were adjusted, so the fluid levels were equal, and, therefore, perfusion pressure delivered to the polyethylene tubing equal. On any given day the perfusion was allowed to run from the time the Krebs-Henseleit solution was prepared until the end of the day. Perfusion bottles were periodically checked to be sure that they did not plug up or run out of fluid. Similarly, temperature in the chamber was periodically determined. The perfusion bottles required refilling about every 45 minutes.

Removal of the tenuissimus muscle required about 15 minutes. The nerve-muscle preparation was placed in the chamber, and the irrigation with Krebs-Henseleit solution begun. Connective tissue was cleared away, and the nerve trunk drawn through the small hole into the adjacent chamber and laid across platinum stimulating electrodes. Test stimuli of 1 every 5 seconds were applied to the nerve to determine threshold voltage for a visible twitch. The polarity on the stimulus isolator was selected to give maximum twitch response. Once this was accomplished, the voltage was increased 2x the level which appeared to elicit maximal response. Pulse duration used was, generally, 50useconds. In a few experiments, pulse durations of 100useconds were used. It was possible to maintain the isolated muscle for 6 or 7 hours without significant decay of resting potentials or development of neuromuscular block. Boyd and Martin (1956b) have maintained tenuissimus muscles for 12 hours.

When a microelectrode is inserted into a muscle fiber in the region of an end plate, small miniature end plate potentials (m.e.p.p.'s) can be observed which are about 0.5mV in amplitude, have a rise time of about 0.5msec, and 1/2 fall of about 1.5msec. M.e.p.p. amplitudes increase, as recording electrodes are positioned



closer to the end plate. Recording at other positions on the fiber either yeild no observations of m.e.p.p.'s, or, on some occasions, m.e.p.p.'s are distorted in shape, are smaller, and have a prolonged half fall.

Three different experiments were carried out measuring m.e.p.p.'s. The first procedure dealt with the effects of NaF in the absence of any blocking drug on the frequency and amplitude of the m.e.p.p.'s.

The oscilloscope was adjusted to a sweep speed of 0.1msec/cm, and the camera adjusted to move continuously at a slow speed (2.5cm/sec). This allows for spreading of the sweep across the width of the film. The width of each sweep was 1 second. Under these conditions, samples were filmed at 1 minute intervals for a duration of 45 seconds. Control samples were taken for 10 minutes prior to introduction of various concentrations of NaF. Resting potential was continuously recorded on the monitor scope and values logged with other relevant information. After 10 minutes of control perfusion, the solution containing NaF was introduced. The responses were monitored and filmed in the same manner for an additional 10 minutes, then the control perfusion restored. Periods of at least 20 minutes were allowed between treatments before each control sample was taken. It was necessary to sample

several cells before the electrode could be positioned in a cell where resting potential changes and other factors, such as noise (60 cycle), were at a minimum throughout the control period.

The second procedure carried out was to obtain data on the time course of the m.e.p.p.'s. To do this, single frames were taken of the m.e.p.p.'s at a sweep speed of 2msec/cm.

In the third experimental procedure the same protocol was followed, except that in the case of these tests, attempts were made to antagonize the postsynaptic blocking effects of d-tubocurarine. Control m.e.p.p.'s were recorded for the usual 10 minute interval and various doses of d-tubocurarine introduced. A concentration of blocking drug was selected which reduced m.e.p.p. amplitude by about 50%. After 10 minute perfusion with d-tubocurarine, NaF was added to the perfusion fluid, and samples of m.e.p.p.'s were taken for an additional 10 minutes. The reverse of this experiment was also carried out. NaF was allowed to act for 10 minutes after the control period, then d-tubocurarine was added to the perfusion.

Two methods were used to analyze the effects of NaF on end plate potentials (e.p.p.'s). In one, the end plate responses were measured in d-tubocurarine

blocked muscle, and, in the other, measurements were made in the cut-fiber preparation of Barstad and Lilleheil (1968).

For investigating the effects of NaF in d-tubocurarine blocked e.p.p.'s, 3ug/ml d-tubocurarine in the Krebs-Henseleit solution was continuously perfused during control and treatment periods. For these experiments the isolated muscle was stimulated indirectly at a frequency of 1 every 5 seconds. Pulse duration was 50-100usec. After about 30-45 minutes of d-tubocurarine perfusion, muscle movement ceases. End plate regions were located by impaling cells in the region of expected nerve endings and monitoring for the presence of e.p.p.'s on an oscilloscope. Once a region of e.p.p.'s was located, the microelectrode was positioned to give optimum rise time and 1/2 fall of 0.5 and 1.5msec respectively. It was observed in our studies as well as the studies of others (Fatt and Katz, 1952; and Boyd and Martin, 1956b) that as the recording electrode was moved along the fiber, the e.p.p. amplitude was decreased and rise time and 1/2 fall increased as the distance from the end plate region increased. In cat tenuissimus muscle, the distance for a 20% reduction in e.p.p. amplitude has been reported by Boyd and Martin (1956b) to average 0.8mm.

E.p.p.'s were recorded in a manner similar to that used for m.e.p.p.'s. The experimental protocol was also restricted to the same time intervals. Control resting potentials were observed for at least 10 minutes before beginning each experiment. At least 15 single frames were taken of a series of elicited e.p.p.'s in the presence of d-tubocurarine. Then the perfusion system was switched to the reservoir containing various concentrations of NaF. 3ug/ml d-tubocurarine was present in both "control" and "treatment" reservoirs. Series of single frames were then taken up to the time transmission was restored, or for 10 minutes in the case of the preparations in which twitching was not restored. The film strips were subsequently developed, and amplitude, rise time, and 1/2 fall measurements were taken from the enlarged displays of each sample as described in the case of the procedures dealing with m.e.p.p.'s.

Effects of NaF in the absence of d-tubocurarine block were obtained by adaptation of the transverse cut-fiber for isolated rat diaphragm, and this method was applied to the cat tenuissimus muscle. This procedure differs from others in that muscle movement is prevented by cutting the cell membranes on either side of the end plate region leaving the muscle fibers to

become depolarized. In this way, muscle action potentials and contractions disappear rapidly due to loss of resting potentials to levels above electrical threshold. Control resting potentials under these conditions normally were 35-45mV compared to the resting potentials of 70-75mV in d-tubocurarine-blocked muscles. The muscle was continuously stimulated indirectly with 50usec duration shocks every 5 seconds. Voltage was adjusted to 2x that required for maximal contraction. A branching nerve was located at a point where it entered the muscle mass, and small transverse cuts were made about 25mm apart across the width of the muscle fiber on each side of the nerve. This was usually sufficient to produce muscle paralysis. However, if movement did not cease, cuts were made closer to the nerve.

End plates were localized in the same manner as described for d-tubocruarine-blocked muscle. Intracellular recordings were obtained for 10 minutes prior to administration of 8mM NaF. During the course of control measurements, single frame photographs were made of end plate responses displayed on the oscilloscope. Similar recordings were made 10 minutes after continuous irrigation of the tissue with 8mM NaF. The film records obtained were subsequently analyzed for amplitude, rise time, and 1/2 fall in the same manner

as in the case of d-tubocurarine-blocked muscle.

The measurement of the quantative components of e.p.p.'s rely on several anatomical, physiological, and statistical assumptions (see discussion). In my studies, I decided to bypass certain of these technical disadvantages inherent in other methods of measuring quantal content and direct my efforts at an indirect method of statistically estimating quantal size ( $q$ ) from the variance of trains of e.p.p.'s. This method is derived originally from the observations of Del Castillo and Katz (1954), of Boyd and Martin (1956b), and of Elmqvist and Quastel (1965).

A muscle preparation was blocked by 3 $\mu$ g/ml d-tubocurarine and an end plate region located in the usual manner. Resting potentials and end plate responses were observed for several minutes. After assuring the muscle was blocked, e.p.p.'s of acceptable time course were recorded. Trains of e.p.p.'s at frequencies of 25/sec or 100/sec were applied to the nerve once each minutes. The train duration was adjusted to elicit 100 stimuli. Photographs of each of the e.p.p.'s were recorded by setting the oscilloscope to a single spot position and running the camera continuously during the stimuli. In this way, amplitude deflections due to e.p.p.'s were recorded. E.p.p. amplitudes of the

10th train of the control period were measured after magnification, along with the 10th train of the e.p.p.'s exposed to drug treatment. Each end plate value was recorded in millivolts. Variance was calculated from the last 80 e.p.p.'s in each train of 100 after correcting for non linear summation according to the following formula:

$$\text{E.p.p.} = \frac{\text{E.p.p.}}{(\text{R.P.}-15-\text{E.p.p.})} \times (\text{R.P.}-15)$$

(Martin, 1955)

Quantal size was estimated from the e.p.p. values as the ratio:

$$q = \frac{\text{variance}}{\text{E.p.p.}}$$

Variance is determined from each train. The value of  $q$  for any set of data is estimated from the variance and mean of the e.p.p.'s in the train. This procedure has been carried out in several ways. The last 80 end plate responses were grouped either in groups of 5, 10, or 40, and the mean end plate response and variance in each group were determined by conventional means:

$$s^2 = \frac{\sum (x - \bar{x})^2}{N-1}$$

A given  $q$  for a sample group was determined as the

ratio of the

$$\frac{\text{variance}}{\text{E.p.p. amplitude}}$$

Each estimate of  $q$  was then summated for the entire train of e.p.p.'s and an average taken to estimate quantal size for the train.

Quantal content ( $M$ ) is calculated for each e.p.p. or for groups of e.p.p.'s from the following relationship (Brown, 1962):

$$x = qM$$

$$\frac{x}{q} = M$$

where  $q$  = quantal size and  $x$  the average of amplitude of a train of e.p.p.'s. In practice,  $M$  is computed for the first 10 or so e.p.p.'s in a train and for the last 80. The first 10 e.p.p.'s and the last 80 e.p.p.'s are called the head values and the tail values respectively. Head values are used for calculation of probability of release ( $P$ ) and readily releasable stores ( $N$ ). The tail values are utilized to estimate the rate of quantal mobilization ( $dm$ ).

In curarized muscle and cut-fiber muscle, e.p.p. potential amplitude characteristically declines during a tetanus, leveling off at a fairly well maintained plateau after about 5 impulses. The initial fall in e.p.p. amplitude is generally considered to be a reflexion of partial depletion by release of the readily



releasable store (Liley and North, 1953; Martin, 1955; and Elmqvist and Quastel, 1965). The level of readily releasable stores (N) for each cell were estimated by determining the summated depletion of quanta during the first few end plate responses. This can be done by visibly fitting a straight line through the linear segment of the depletion curve plotted as quantal content (M) on the ordinate versus cumulative depletion on the abscissa. The extrapolated intercept on the abscissa reflects releasable stores as the quanta released during linear depletion. A similar value can be approximated by fitting a regression line through the first few e.p.p.'s of a train by the method of least squares. The linear portion of the regression line is determined by establishing the point at which the theoretical line deviates from the observed values. This can be accomplished by measuring changes in correlation coefficients calculated for each point.

Probability of release (P) is determined as the ratio of the quantal content of the first e.p.p. (M) to the level of readily releasable stores (N) estimated from the regression line:

$$P = \frac{M}{N}$$

This value can be determined from the data pro-

duced above. Blaber (1970) has related probability of release to the slope of the quantal content depletion curve compiled for calculation of N.

Mobilization (dm) was calculated from the mean quantal content of the tail values after the method of Blaber (1970). This is described as the mean quantal content of the tail divided by the stimulus interval.

The above procedures were also utilized in the cut-fiber preparation to assess the effects of NaF in the absence of d-tubocurarine. In these experiments the methods used were identical save for the fact that stimulation frequency had to be adjusted to 200 per sec in order to obtain successive depletion of the first few e.p.p.'s (Blaber, 1970).

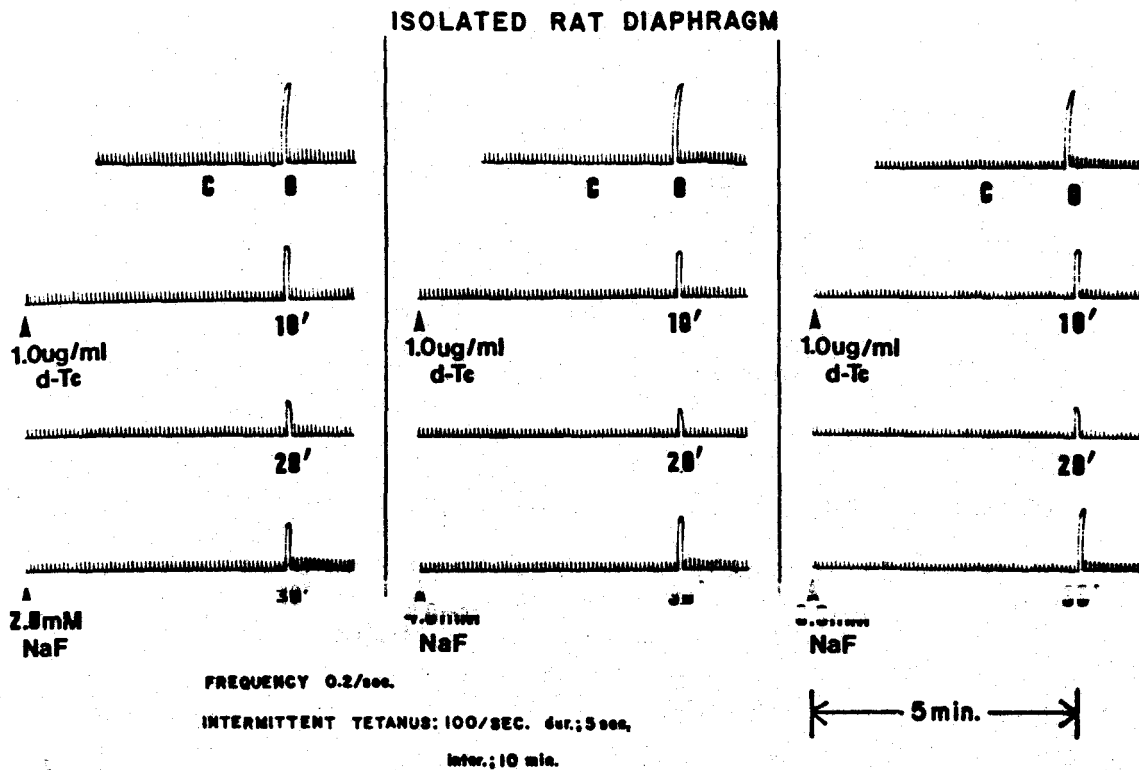
## RESULTS

Mechanical muscle movement. - Several experiments were carried out to investigate the action of NaF on mechanical muscle movement. In these preliminary tests I attempted to compare the effects of fluoride on rat diaphragm and kitten diaphragm, then on cat tenuissimus muscle. I was interested in knowing whether in all three preparations an anticurare action of NaF could be realized. Further, I wished to establish a dose level in cat tenuissimus which was clearly anticurare like in action in order to select a pharmacologically relevant concentration for study with intracellular microelectrode methods.

Figure 2 represents sample recordings selected from a series of 3 experiments in isolated rat diaphragm in which the effects of fluoride were tested in the absence of any neuromuscular blocking drugs. In all three experiments, there appeared to be a gradual deterioration of twitch height during the course of the experiment, similar to that described by Blaber (1970) from his studies with rat diaphragm.

In figure 2 are three control periods obtained in the same preparation. Individual twitch heights were depressed between the control period for the 2mM NaF

FIGURE 2



Effect of NaF on the isolated rat diaphragm. Recordings are sample traces from a single preparation. Control twitch heights were obtained by indirect stimulation of the phrenic nerve at a frequency of 12/min. Tetanus responses shown result from indirect stimulation at a frequency of 100/sec. Control recordings are indicated by zero time (0'). Effects of fluoride are shown at 10', 20', and 30' after addition to the bath.

dose and the control period for the 8mM NaF dose. Also depressed were the responses to tetanic stimulation although to a somewhat lesser extent. Post tetanic responses similarly showed suppression. These results suggest that the effects of various doses of fluoride may accumulate, or, on the other hand, a significant degree of the depression may be due to gradual deterioration of the preparation.

For studying the effects of individual doses of NaF on neuromuscular transmission, I compared the response obtained at zero time with responses obtained after 5, 15, and 25 minutes of continuous exposure to 2, 4, and 8mM NaF.

At the 2mM concentration, NaF produced verly little effect on the pre tetanus twitch height, tetanus height, or post tetanus twitch height, although the first post tetanic response appeared slightly depressed after 35 minutes of drug exposure. At 4mM NaF as well as at 8mM NaF pre tetanus twitch height was clearly depressed as was tetanus height and post tetanic responses.

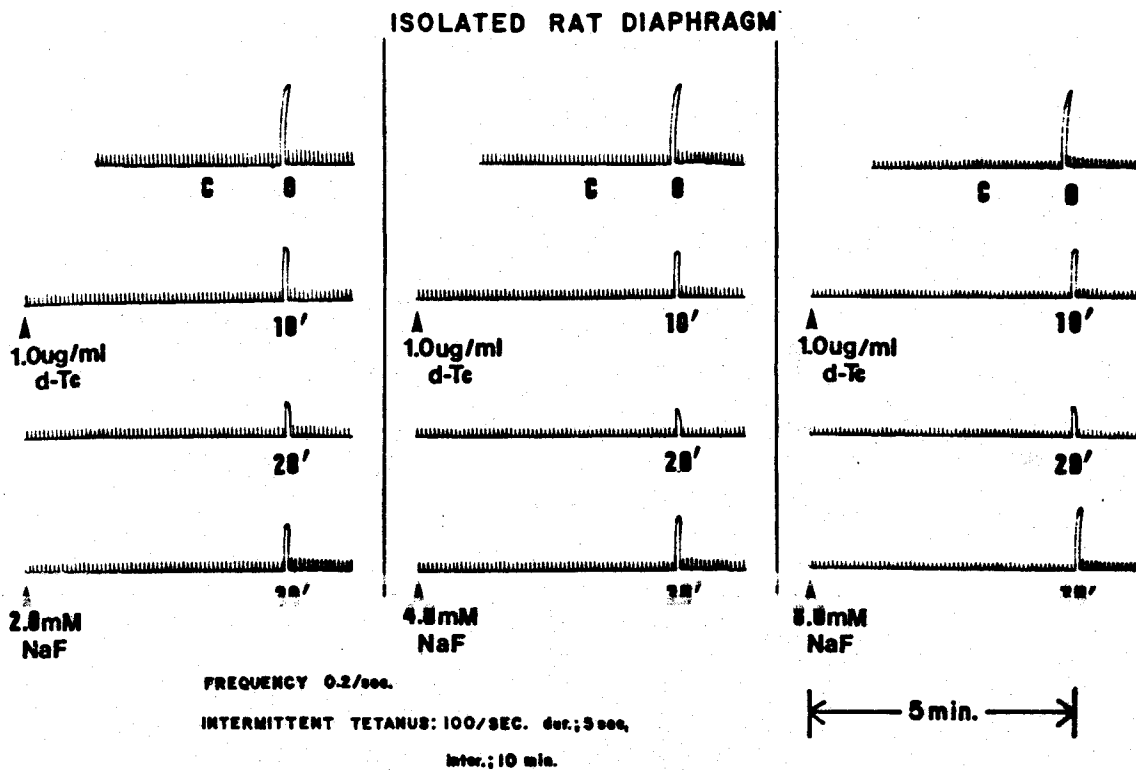
The results obtained in our tests were qualitatively similar to those reported by Blaber (1970) with NaF. In our study and his, concentrations of 2, 4, and 8mM NaF did not appear to facilitate neuromuscular transmission. In fact, a slight but discernable neuro-

muscular depression became evident. It is interesting to note that the time course of the tetanic contraction appeared unaltered in these studies. If acetylcholinesterase were inhibited, one would expect a shortening of the contracture time and depression of contracture height characteristic of treatment with neostigmine (Blaber and Bowman, 1963).

The neuromuscular depression seen in the rat diaphragm with fluoride is not similar to a d-tubocurarine block. The latter can be expected to be somewhat reversed by tetanus, and this was not the case with NaF-induced block. As can be seen in figure 2 intermittent tetanus (100/sec, dur:5sec) applied every 10 minutes had no effect on the gradual depression produced by fluoride. It may be that NaF exerts a weak hemicholinium like action, in that the responses obtained (i.e. gradual reduction in twitch height) were qualitatively similar to that seen with hemicholinium-3 (Long and Schueler, 1954).

Figure 3 represents the results of 3 experiments in isolated rat diaphragm in which the anticurare action of NaF were assessed. The figure shown represents the results of a single experiment in which 2, 4, and 8mM NaF were tested against 1ug/ml d-tubocurarine. The test conditions and protocol used were similar to conditions used to test the effects of NaF in the absence

FIGURE 3



Effect of NaF on d-tubocurarine blocked rat diaphragm. Recordings are sample traces from a single preparation. C indicates a control period in which no drugs were given. d-Tc and NaF indicate the points in time when d-tubocurarine and NaF were added to the bath.

of neuromuscular blockade. Examination of the control data for each dose shows that there occurred a gradual deterioration of pre tetanus height, tetanus height, and the post tetanic responses over the course of the experiment. It is not known whether this deterioration represents a blocking action of NaF or not.

A slight neuromuscular block was produced 20 minutes prior to administration of each dose of fluoride by adding d-tubocurarine to the bath (1.0ug/ml final concentration). This concentration of d-tubocurarine produced a 25% reduction in pre tetanus height after 15 minutes of exposure. Tetanus height was reduced 50% and post tetanic response about 25%. Addition of 2mM NaF, after 20 minutes exposure to d-tubocurarine, resulted in only a very slight antagonism of d-tubocurarine. Tetanus height was 60% of the control in the presence of 2mM NaF as compared to 50% of the control height for the tetanus response preceding the 2.0mM dose.

The test of 4mM NaF showed an increased antagonism of d-tubocurarine. In this case d-tubocurarine reduced pre tetanus height only slightly, whereas the tetanic response was reduced to 30% of control. Like the pre tetanus height, the post tetanic response was only slightly depressed by d-tubocurarine. 4mM NaF



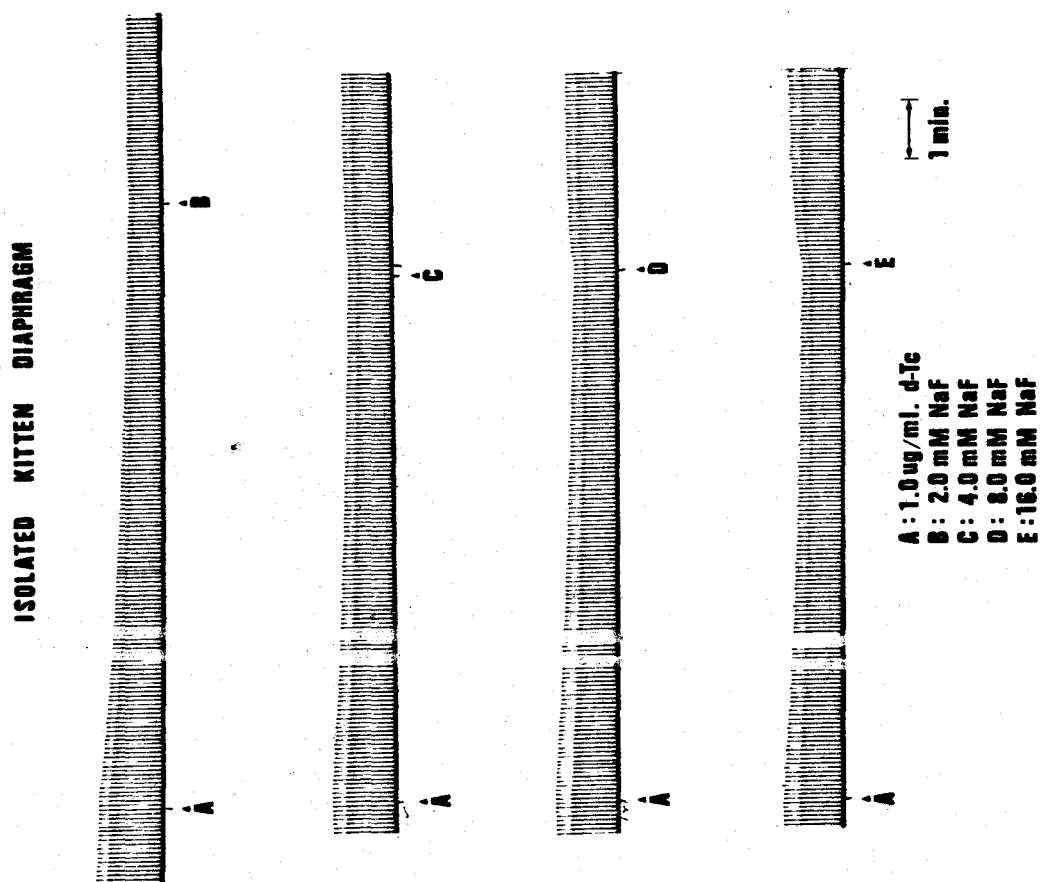
antagonized the depressant effect of d-tubocurarine by restoring tetanus height to 70% of control. Pre and post tetanic responses seemed little affected.

8mM NaF antagonized the d-tubocurarine depressed tetanic response to a greater extent that obtained with either 2 or 4mM NaF. In this test, tetanic height was depressed to about 40% of control by d-tubocurarine. Addition of 8mM NaF reversed d-tubocurarine to an extent where tetanus height was about 80% control height. At this concentration of fluoride pre tetanic and post tetanic responses were not altered by fluoride.

In the three experiments undertaken, other doses of d-tubocurarine were tried but did not yield additional useful information other than the established fact that a higher dose of d-tubocurarine produced an increasing degree of block.

Figure 4 represents an example of results obtained from isolated kitten diaphragm experiments. Three experiments were carried out in all. Stimulation frequency in these studies was 0.2/sec as in the isolated rat diaphragm studies. Intermittent tetanus was not included in these tests, as it was apparent that 1ug/ml d-tubocurarine clearly depressed neuromuscular transmission after about 10 minutes of exposure. As can be seen in this figure, increasing

FIGURE 4



Anti-curare effect of NaF on isolated kitten diaphragm. Twitch responses were obtained by indirect stimulation of the phrenic nerve at a frequency of 100/sec. A indicates the addition of d-tubocurarine to the bath. B,C,D,E indicate addition of various doses of NaF.

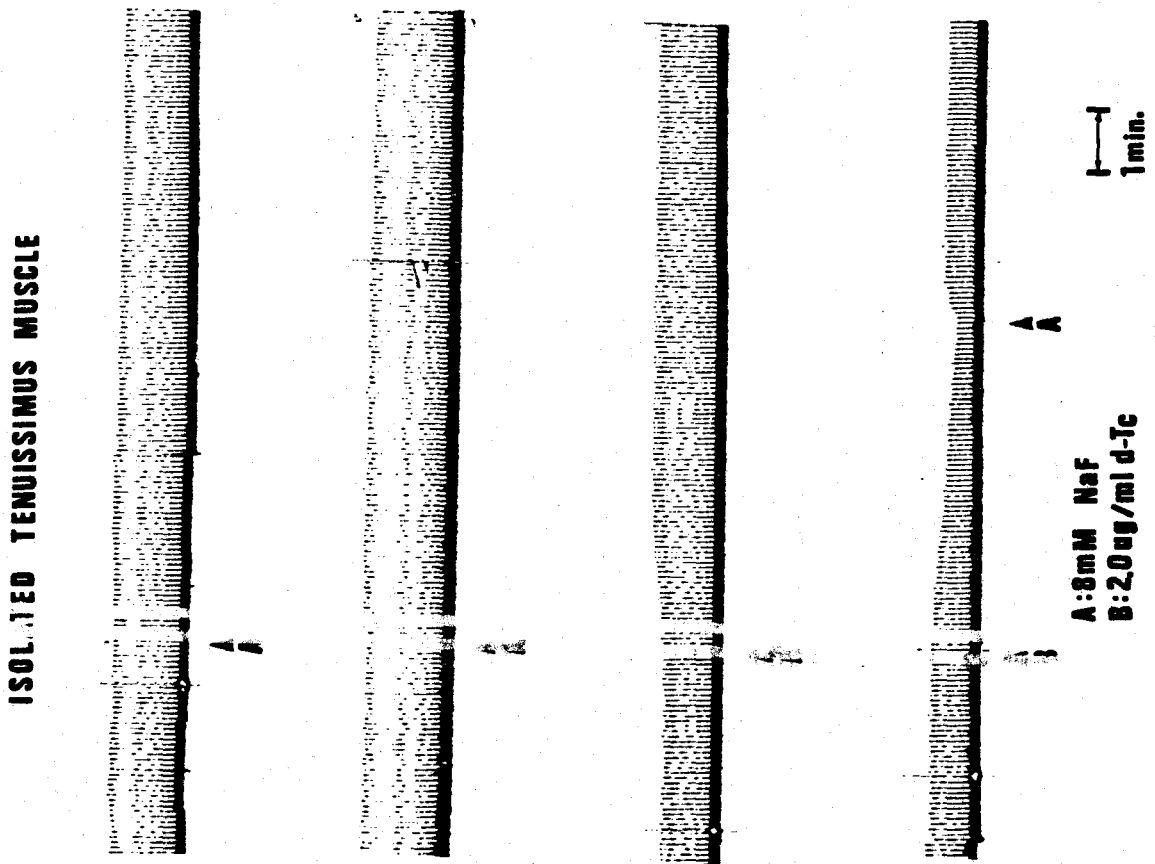
concentrations of NaF appeared to reverse progressively the d-tubocurarine block.

2mM NaF appeared to have little effect, whereas 4, 8, and 16mM NaF provided corresponding increases in the degree of d-tubocurarine reversal. These data suggested that NaF is equally effective in the rat or cat diaphragm.

The neuromuscular blocking action of NaF seen in the rat diaphragm (Blaber, 1970) and in our test (figure 2) was not evident in the kitten diaphragm. This lack of depressant action or gradual deterioration may possibly be associated with the fact that we deleted the intermittent tetanus from these studies. Therefore, from a negative point of view, this suggests that the NaF blocking action may be realized at high frequency rather than at low frequency stimulation.

Figure 5 demonstrates the twitch responses obtained from three experiments utilizing isolated cat tenuissimus muscle. Stimulation frequency was 0.2/sec, the same frequency as used for rat and kitten diaphragm. As with the kitten diaphragm, intermittent tetanus was omitted from these experiments. The first three tracings shown in figure 5 are from separate cats showing the effects of exposure of the muscle to 8mM NaF in the absence of any neuromuscular blocking drug.

FIGURE 5



Effect of NaF in the presence and absence of d-tubocurarine in isolated cat tenuissimus muscle. A indicates the addition of 8mM NaF. The first three tracings are from 3 separate cats. The fourth tracing demonstrates an insuing paralysis occurring after addition of d-tubocurarine (B) and its reversal after addition of 8mM NaF (A).

The last tracing in the figure demonstrates the anti-curare action of NaF.

Examination of the first three tracings shows that NaF possesses little blocking action of the type noted in the rat diaphragm. It was observed that facilitation, indicated by an increased twitch height, was evident in one preparation but could not be demonstrated in the others. The anticurare action of NaF was uniformly reproduced in all studies at a concentration of 8mM.

Comparing our studies with the studies of Koketsu and Gerard (1956), it is interesting to note that the latter authors observed that muscle fasciculations commonly occurred in frog muscle treated with 1-10mM fluoride. We did not observe fasciculations in our studies with rat or cat diaphragm or with cat tenuissimus muscle. Koketsu and Gerard (1956) attributed the fibrillatory action of fluoride in frog muscle to the occurrence of m.e.p.p.'s that were large enough to spontaneously initiate muscle action potentials. Although this may occur in the absence of a d-tubocurarine block, the relationship between fibrillation and an anticurare action of fluoride is not clear.

Two other interesting differences arise between the effects of fluoride on mechanical response in frog

and cat muscle. Koketsu and Gerard stated that "no sign of blocking of nerve-muscle transmission was ever observed". In contrast, Blaber (1970) noted clear blocking actions in rat diaphragm. In my studies I have noted this blocking effect only in studies utilizing intermittent tetanus. Koketsu and Gerard applied stimuli at a low frequency (0.2/sec) at intervals of 5 minutes. In my studies I used the same frequency stimulation; however, pulses were applied continuously. Thus, from these results it is difficult to ascribe the blocking action of fluoride to only a difference in species, since it may be dependent in part on the frequency of stimulation used.

The second point of interest that relates to differences between frog and cat muscle is the response of the muscle to tetanus. In frog muscle, when strong repeated tetanization was applied, the subsequent single muscle responses were depressed. NaF augmented or partially reversed this post tetanic depression (Koketsu and Gerard, 1956). In Blaber's studies (1970) deterioration of twitch and tetanus height was intensified by 8mM NaF. This is also evident in my studies (figure 2).

The fact that NaF has anticholinergic properties in the frog, rat, cat, and dog suggests that the mechanism

of the action of fluoride might not be different in the various species.

Miniature end plate potentials (m.e.p.p.'s). -

The action of fluoride on m.e.p.p.'s occurring in cat tenuissimus muscle was undertaken to investigate transmitter release, postsynaptic sensitivity, and time course of the m.e.p.p. An additional series of experiments were undertaken to determine the effects of d-tubocurarine on the amplitude of m.e.p.p.'s and to evaluate the degree to which the postsynaptic depressant action of d-tubocurarine could be reversed by NaF.

Table 1 represents the results of 4 experiments in which the effects of 2mM NaF on m.e.p.p. amplitude were investigated. This data represents samples of miniatures filmed prior to drug treatment (control) and 10 minutes after exposure to 2mM NaF. In all experiments there occurred a slight decrease in m.e.p.p. amplitude after treatment with fluoride. In three experiments the mean amplitude measurements were not significantly different between the control and 2mM NaF. In one experiment the m.e.p.p. amplitude decreased to a level which was statistically significant from controls. These data are in striking contrast to what is obtained in frog muscle. In the latter

TABLE 1

Effect of 2mM NaF on m.e.p.p. Amplitude

<u>Experiment</u>	<u>Treatment</u>	<u>N</u>	<u>Amplitude (mm)<sup>+</sup>s.d.</u>	<u>t</u>
I	Control	196	7.331 $\pm$ 2.137	
	Treat	158	6.949 $\pm$ 1.874	1.760
II	Control	122	5.024 $\pm$ 1.534	
	Treat	87	3.563 $\pm$ 1.053	7.649*
III	Control	34	9.735 $\pm$ 2.744	
	Treat	79	9.379 $\pm$ 2.548	0.659
IV	Control	13	5.076 $\pm$ 1.114	
	Treat	39	4.871 $\pm$ 0.4185	

---

\* Statistically significant difference between control and treated responses ( $P < 0.05$ ).



TABLE 2

Effect of 4mM NaF on m.e.p.p. Amplitude

<u>Experiment</u>	<u>Treatment</u>	<u>N</u>	<u>Amplitude (mm) <math>\pm</math> s.d.</u>	<u>t</u>
I	Control	174	6.212 $\pm$ 1.992	
	Treat	224	8.482 $\pm$ 2.350	10.182*
II	Control	32	9.812 $\pm$ 2.022	
	Treat	25	11.200 $\pm$ 2.449	2.301*

---

\* Statistically significant difference between control and treated responses ( $P < 0.05$ ).

TABLE 3

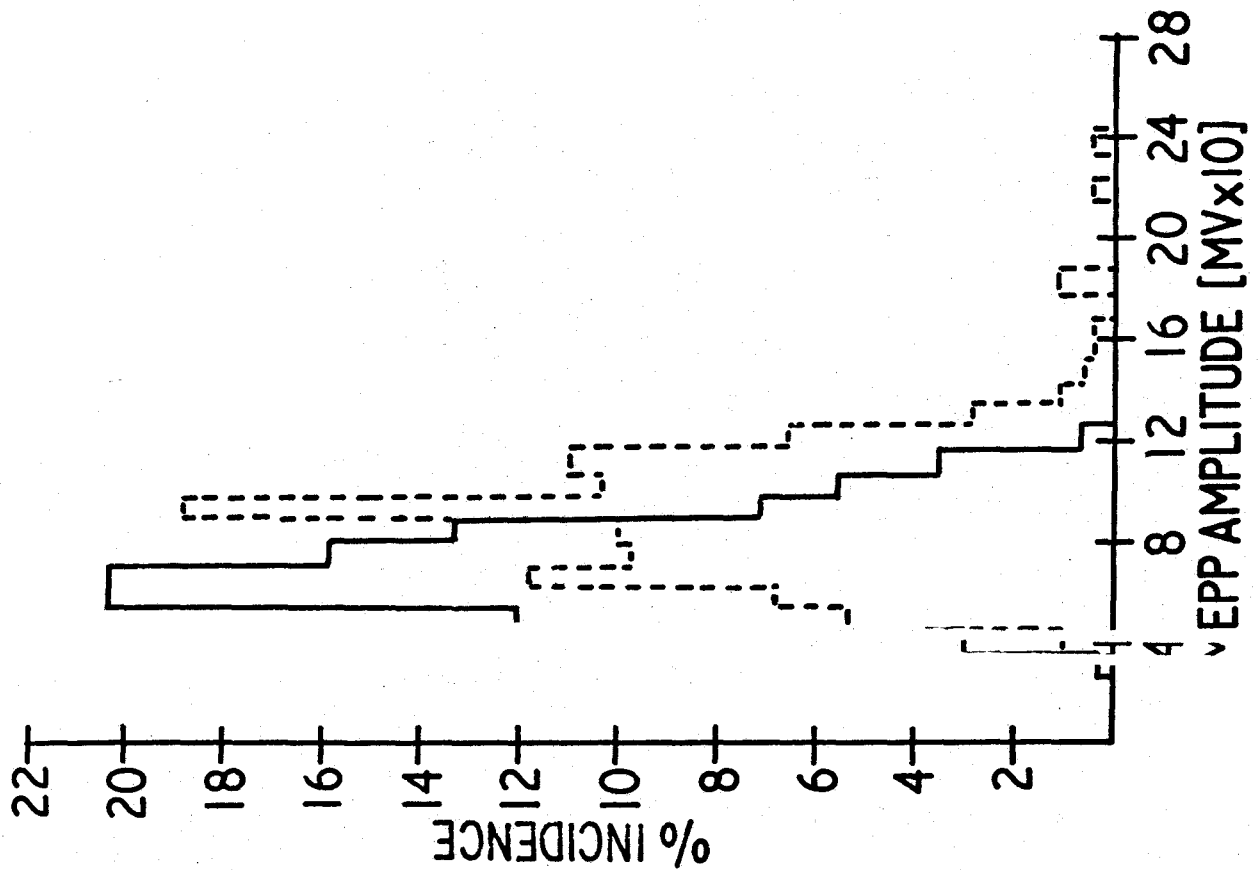
Effect of 8mM NaF on m.e.p.p. Amplitude

<u>Experiment</u>	<u>Treatment</u>	<u>N</u>	<u>Amplitude (mm)<sup>†</sup>s.d.</u>	<u>t</u>
I	Control	127	7.149 $\pm$ 1.927	
	Treat	191	7.408 $\pm$ 1.954	1.168
II	Control	79	9.050 $\pm$ 2.269	
	Treat	140	9.921 $\pm$ 2.481	2.560*
III	Control	221	7.162 $\pm$ 1.928	
	Treat	369	11.135 $\pm$ 3.09	17.188*

---

\* Statistically significant difference between control and treated responses ( $P < 0.05$ ).

FIGURE 6



Effect of 8mM NaF on m.e.p.p. amplitude. Control distribution of m.e.p.p.'s are indicated by solid lines. Broken lines indicate distribution of m.e.p.p.'s obtained after 10 minute perfusion with 8mM NaF.

case, concentrations of 0.5 to 10mM increased m.e.p.p. amplitude. 3mM NaF produces m.e.p.p.'s nearly 3x that obtained in the controls (Koketsu and Gerard, 1956).

Table 2 indicates the effects of 4mM NaF on m.e.p.p. amplitude. In this case significant increases in m.e.p.p. amplitude were realized. The magnitude of response ranged from a 13% to a 30% increase.

The effect of 8mM NaF are demonstrated in table 3 and figure 6. In 2 of the 3 experiments 8mM NaF resulted in a slight but statistically significant increase in m.e.p.p. amplitude. Attendant with this concentration was a small incidence of very large m.e.p.p.'s ranging from 1.6mV to as much as 2.4mV (figure 6). These large m.e.p.p.'s were rarely seen during control periods. In calculation of the mean m.e.p.p. amplitude they were excluded since they may produce some bias in assessing the postsynaptic effects of fluoride.

Tables 4, 5, and 6 summarize the effects of NaF on m.e.p.p. frequency/sec at test concentrations of 2, 4, and 8mM NaF.

At 2mM NaF (table 4) mixed effects were seen. In 2 experiments frequency increased significantly. In the other 2 experiments, there was no effect in one and a significant decrease in m.e.p.p. frequency

TABLE 4

Effect of 2mM NaF on m.e.p.p. Frequency/sec

<u>Experiment</u>	<u>Treatment</u>	<u>N</u>	<u><math>\bar{f} \pm</math> s.d.</u>	<u>t</u>
I	Control	25	2.765 $\pm$ 0.453	
	Treat	24	2.516 $\pm$ 0.515	1.762
II	Control	33	1.921 $\pm$ 0.360	
	Treat	28	1.665 $\pm$ 0.586	2.058*
III	Control	29	0.936 $\pm$ 0.337	
	Treat	28	1.484 $\pm$ 0.797	3.341*
IV	Control	39	0.369 $\pm$ 0.553	
	Treat	27	0.896 $\pm$ 0.764	3.201*

---

\* Statistically significant difference between control and treated responses ( $P < 0.05$ ).

TABLE 5

Effect of 4mM NaF on m.e.p.p. Frequency/sec

<u>Experiment</u>	<u>Treatment</u>	<u>N</u>	<u><math>\bar{f} \pm \text{s.d.}</math></u>	<u>t</u>
I	Control	23	2.651 $\pm$ 0.459	
	Treat	27	2.794 $\pm$ 0.501	1.027
II	Control	26	1.038 $\pm$ 0.439	
	Treat	28	0.808 $\pm$ 0.459	1.849

---

\* Statistically significant difference between control and treated responses ( $P < 0.05$ ).

in the other. The frequency data were determined from the same m.e.p.p.'s in which amplitude measurements were made (table 2). It is interesting to note that at this concentration, there appeared to be no relationship between changes in m.e.p.p. frequency and changes in m.e.p.p. amplitude.

At 4mM NaF (table 5) similar results on m.e.p.p. frequency were obtained as that seen with 2mM NaF. In one experiment a slight increase in frequency was realized; in the other a slight decrease in frequency occurred. These data were not statistically significant.

Table 6 shows the effect of 8mM NaF on m.e.p.p. frequency. In this case a uniform increase occurred in all three experiments. This modest but uniform effect was statistically significant. Figure 7 shows the distribution of the pooled frequency data collected for the 8mM NaF experiments. The distribution is plotted as the incidence (ordinate) versus the m.e.p.p.'s occurring in 1 second of sample time (abscissa). As can be seen in the figure, the highest incidence recorded in the controls occurred at a frequency of 3 m.e.p.p.'s/sec. 8mM fluoride shifted the peak incidence to about 4 m.e.p.p.'s/sec. Perhaps what can be called a unique occurrence was a small incidence of

TABLE 6

Effect of 8mM NaF on m.e.p.p. Frequency/sec

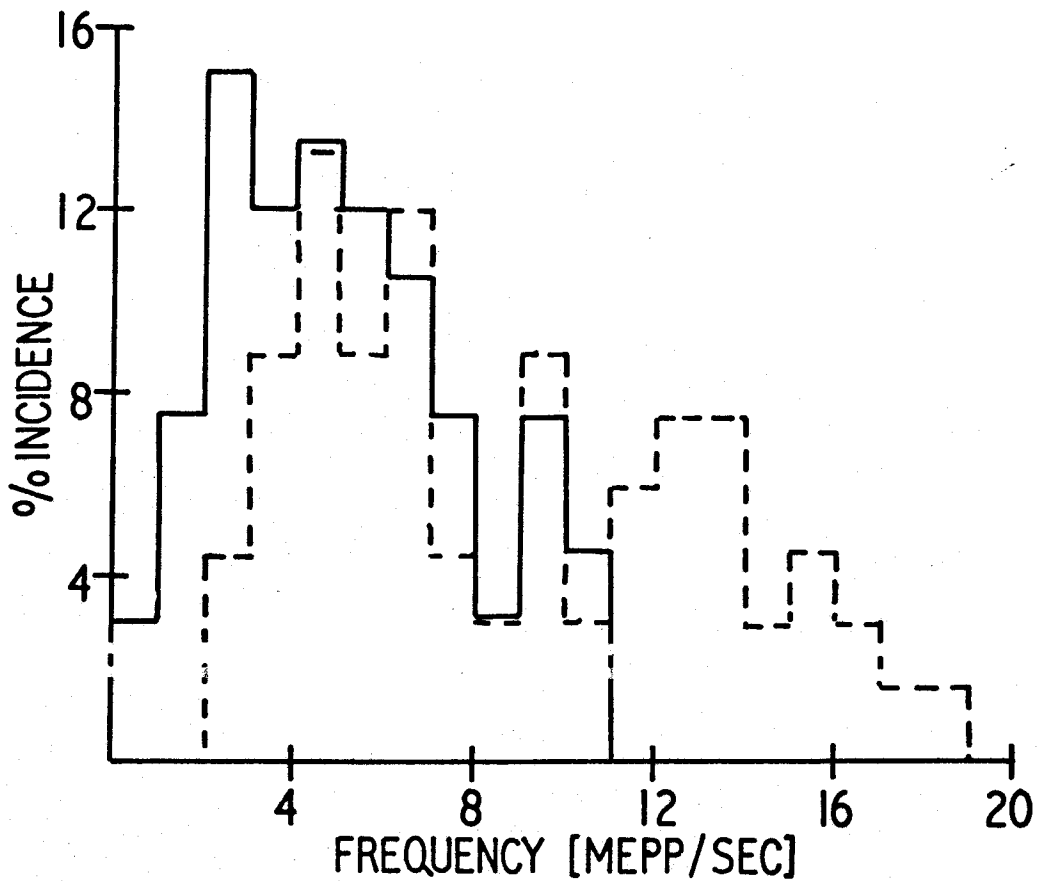
<u>Experiment</u>	<u>Treatment</u>	<u>N</u>	<u><math>\bar{f} \pm \text{s.d.}</math></u>	<u>t</u>
I	Control	28	2.038 $\pm$ 0.612	
	Treat	24	2.734 $\pm$ 0.596	4.145*
II	Control	29	1.598 $\pm$ 0.553	
	Treat	31	2.076 $\pm$ 0.403	3.779*
III	Control	26	2.838 $\pm$ 0.409	
	Treat	28	3.586 $\pm$ 0.529	5.6734*

---

\* Statistically significant difference between control and treated responses ( $P < 0.05$ ).



FIGURE 7



Effect of 8mM NaF on m.e.p.p. frequency/sec. Control distribution of m.e.p.p.'s are indicated by solid lines. Broken lines indicate distribution of m.e.p.p.'s obtained after 10 minute perfusion with 8mM NaF.

relatively high frequency bursts ranging from 12 m.e.p.p.'s/sec to 19 m.e.p.p.'s/sec. These "bursts" did not occur with any rhythm nor did they occur during a particular period during the sample time.

Upon analyzing the m.e.p.p. data, it appeared to me, that although there was a slight increase in m.e.p.p. amplitude at 4mM, these results did not correspond in magnitude to the marked increase in amplitude characteristic of the frog neuromuscular preparation. Very large m.e.p.p.'s were observed only at 8mM NaF coincident with an increase in frequency. Due to the altered discharge rate and the appearance of "giant" miniatures occurring at 8mM NaF, I was not able to assign a clear presynaptic or postsynaptic action of fluoride in cat tenuissimus. The data obtained at 4mM NaF suggested a weak postsynaptic action of fluoride (i.e. only m.e.p.p. amplitude increased); whereas at 8mM, a presynaptic component comes into play (i.e. m.e.p.p. frequency increased).

The question of importance to me was whether the fluoride induced postsynaptic increase in m.e.p.p. amplitude would be of sufficient magnitude to reverse the paralyzing action of d-tubocurarine. In order to investigate this point, a dose of d-tubocurarine was selected that would partially depress m.e.p.p. amplitude.

Doses of 3.0ug/ml, 1.0ug/ml, and 0.5ug/ml progressively depressed m.e.p.p. amplitude to a level where they were no longer detectable on the oscilloscope. Perfusion of 8mM NaF for 10 minutes did not reverse the action of any of these concentrations of d-tubocurarine. At a concentration of 0.25ug/ml d-tubocurarine, it was found that m.e.p.p. amplitude was depressed significantly but not to a level where measurements could not be made.

Table 7 contains the results of 6 experiments in which the effects of 8mM NaF on d-tubocurarine depressed m.e.p.p.'s were shown. Experiment I and II are from different cells of the same cat. Experiment III and IV represent two different cells from another cat and experiment V and VI results from the cells of a third cat.

In these experiments, control mean m.e.p.p.'s amplitudes were calculated from 30 one second samples taken immediately prior to addition of 0.25ug/ml d-tubocurarine to the perfusion. Mean m.e.p.p. amplitudes for the 0.25ug/ml d-tubocurarine treatment were determined from 30 one second samples after 10 minutes of perfusion. The treatment indicated by 0.25ug/ml d-tubocurarine and plus 8mM NaF is the mean m.e.p.p. amplitude 10 minutes after addition of 8mM NaF to the

TABLE 7

Effect of 8mM NaF on d-tubocurarine depressed m.e.p.p.'s

<u>Experiment</u>	<u>Treatment</u>	<u>N</u>	<u>Amplitude (mV) <math>\pm</math> s.e.</u>
I	Control	39	0.6705 $\pm$ 0.0266
	0.25ug d-Tc	56	0.3835 $\pm$ 0.0131*
	0.25ug d-Tc + 8mM NaF	55	0.3300 $\pm$ 0.0109*
II	Control	64	0.4890 $\pm$ 0.0196
	0.25ug d-Tc	94	0.2372 $\pm$ 0.0053*
	0.25ug d-Tc + 8mM NaF	141	0.3308 $\pm$ 0.0068*
III	Control	96	0.8385 $\pm$ 0.0265
	0.25ug d-Tc	96	0.5797 $\pm$ 0.0186*
	0.25ug d-Tc + 8mM NaF	129	0.5018 $\pm$ 0.0102*
IV	Control	96	0.5953 $\pm$ 0.0217
	0.25ug d-Tc	131	0.5311 $\pm$ 0.0134*
	0.25ug d-Tc + 8mM NaF	101	0.3119 $\pm$ 0.0102*
V	Control	69	0.7191 $\pm$ 0.0187
	0.25ug d-Tc	57	0.7217 $\pm$ 0.0244
	0.25ug d-Tc + 8mM NaF	50	0.5723 $\pm$ 0.0211*
VI	Control	39	0.8481 $\pm$ 0.0304
	0.25ug d-Tc	50	0.5531 $\pm$ 0.0256*
	0.25ug d-Tc + 8mM NaF	47	0.5761 $\pm$ 0.0288*

\* P 0.05 relative to control.

perfusion computed in an identical manner.

In each test, with the exception of experiment V, 0.25ug/ml d-tubocurarine significantly depressed m.e.p.p. amplitude. The degree of depression averaged about 27%. The smallest degree of depression occurred in experiment IV (19% depression) and the highest degree of depression occurred in experiment II (52% depression).

Perfusion of the muscle with a solution containing 0.25ug/ml d-tubocurarine and 8mM NaF for 10 minutes failed to reveal any increase in m.e.p.p. amplitude with the exception of experiment II where a slight reversal seemed to occur.

All of the data contained in table 7 were pooled and summarized in table 8. As can be seen in this table, 8mM NaF appeared unable to reverse the depressant effect of d-tubocurarine.

If I assume that the amplitude of the m.e.p.p. is an indicator of the sensitivity of the end plate region to transmitter, and that the action of d-tubocurarine is directed to antagonizing competitively ACh, then the postsynaptic sensitizing action of NaF would be expected to perhaps reverse the d-tubocurarine effect. This did not occur in my studies. Analysis of the data shows that the amplitude of the m.e.p.p.

TABLE 8

Effect of 8mM NaF on d-tubocurarine depressed m.e.p.p.'s  
(summary table)

<u>Treatment</u>	<u>N</u>	<u>Mean Amplitude<sup>±</sup>s.e.</u>
Control (A)	403	0.6934 <u>±</u> 0.0570
0.25ug d-Tc (B)	484	0.5010 <u>±</u> 0.0688
0.25ug d-Tc (C) + 8mM NaF	523	0.4372 <u>±</u> 0.0517
A vs B	t=2.1119	*P < 0.05
B vs C	t=0.6030	*P > 0.05
A vs C	t=2.468	*P < 0.05

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\*P < 0.05 is considered a statistically significant difference between control and treated groups.

was further depressed by the addition of 8mM NaF. This depression may be due to progressive effects from continuous perfusion with d-tubocurarine rather than due to an action of NaF.

The interaction of the sensitizing effect of fluoride with d-tubocurarine was carried one step further. Table 9 represents the results of a single experiment in which the sensitizing action of fluoride was shown to be blocked out by addition of 0.25ug/ml d-tubocurarine to the perfusate.

In summary, it becomes apparent that the post-synaptic depression of d-tubocurarine as indicated by the reduced m.e.p.p. amplitude was not antagonized by 8mM NaF. Further, the sensitizing effect of NaF on m.e.p.p. amplitude, seen when fluoride was tested alone, was abolished by 0.25ug/ml d-tubocurarine. Of interest is the fact that these studies on mechanical movement of cat tenuissimus muscle demonstrate that at a concentration of 8mM, NaF reverses a d-tubocurarine induced neuromuscular block.

Table 10 shows the osmotic influence of 8mM NaF on the Krebs-Ringer utilized in these studies. By calculation, it was determined that 8mM NaF should contribute about 16mOsm/Kg to our Krebs solution. Direct measurement by "freezing point determination" showed

TABLE 9

Effect of d-tubocurarine on NaF "sensitized" m.e.p.p.'s

<u>Treatment</u>	<u>N</u>	<u>Mean Amplitude<sup>±</sup>s.e</u>
Control (A)	72	0.4322 <sub>-</sub> 0.0428
8mM NaF (B)	111	0.7386 <sub>-</sub> 0.0189
8mM NaF + 0.25ug d-Tc (C)	189	0.3565 <sub>-</sub> 0.0067
A vs B	t=7.436	*P < 0.05
B vs C	t=22.5162	*P < 0.05
A vs C	t=2.6419	*P < 0.05

---

\*P < 0.05 is considered a statistically significant difference between control and treated groups.



TABLE 10

Effect of 8mM NaF on osmotic strength of  
Krebs-Ringer solution

<u>Tube</u>	<u>Reading (mOsm/Kg)</u>
standard (100mOsm)	99
standard (500mOsm)	495
Krebs-Ringer	280
Krebs-Ringer + 8mM NaF	291

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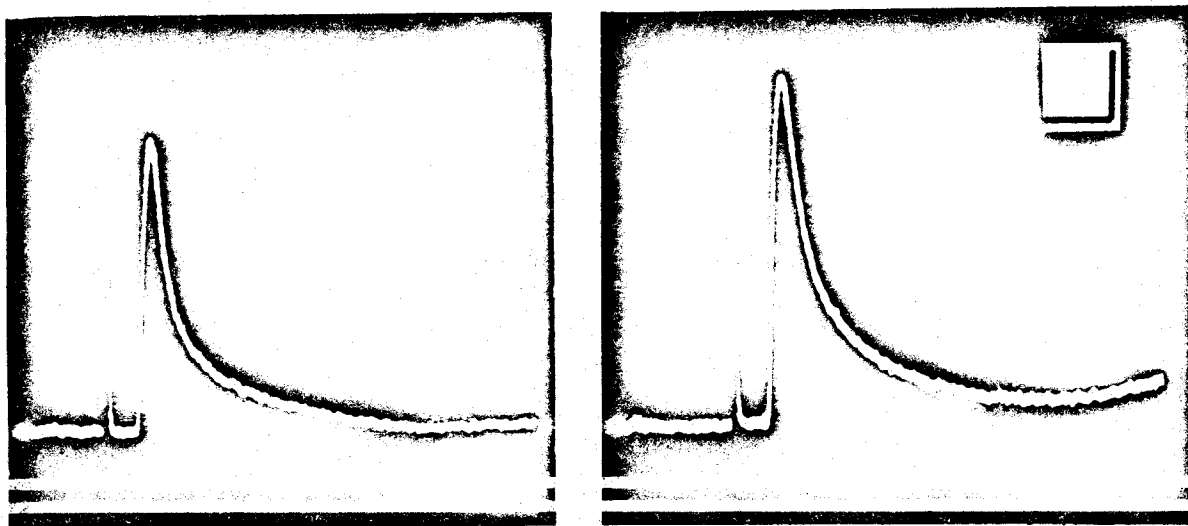
Osmotic strength determined by freezing point depression.

that this concentration of fluoride raised osmotic strength from 280mOsm to 291mOsm or a net gain of 11 mOsm. This constitutes an increase in osmotic strength of about 4%. Although it is possible that this may affect m.e.p.p.'s, this increase in osmotic strength falls within our laboratory error of preparing Krebs-Ringer solution; and therefore, osmotic effects may be difficult to be uniformly demonstrated.

Amplitude and time course of the e.p.p. - Two basic procedures were carried out utilizing intracellular microelectrode measurements. In the first procedure, cat tenuissimus muscle contractions were completely blocked by prior perfusion of 3ug/ml d-tubocurarine. End plate regions were located by intracellular microelectrode methods and the recorded e.p.p. were displayed on an oscilloscope. In the second procedure, e.p.p.'s were recorded in the same manner in cat tenuissimus muscles in which contractions were prevented by cutting the muscle fibers transversely with a fine scalpel.

Figure 8 contains two e.p.p.'s taken from a single experiment in a d-tubocurarine blocked muscle. The left hand panel shows the control e.p.p., and the right hand panel an e.p.p. recorded in the presence of 8mM

FIGURE 8



E.p.p.'s recorded in d-tubocurarine ( $\mu\text{g/ml}$ ) blocked muscle. Left panel=control; right panel=8mM NaF. Calibration=1mVx2msec; stimulation interval=0.2sec.

NaF just prior to restoration of muscle movement.

As can be seen in the illustration, the e.p.p. is of greater amplitude in the right hand panel than in the left hand panel, thus indicating an anticurare action of 8mM NaF. Rise time is slightly increased while 1/2 fall appears little affected. The change in shape of the membrane potential, shown in the right panel which occurred in the last phase of the e.p.p. decline, is probably due to muscle movement.

Table 11 summarizes the pooled data from four experiments in which groups of e.p.p.'s obtained during the control period and 10 minutes after administration of 8mM NaF were compared. It should be noted that after beginning NaF perfusion, the e.p.p.'s monitored on the oscilloscope began to increase progressively in amplitude. In most experiments attempted, neuromuscular transmission was restored 10 minutes after treatment with fluoride. The e.p.p.'s used for comparison were those occurring during the last one or two minutes prior to the restoration of muscle movement. These e.p.p.'s were compared with e.p.p.'s occurring in the last two minutes of the control period. In the first experiment, 25 control e.p.p.'s were obtained and compared with seven treated e.p.p.'s. In the second experiment, 25 controls were compared with

TABLE 11

Effect of 8mM NaF on e.p.p.

Experiment & Treatment	N	Mean Amplitude (cm) <sup>±</sup> s.d.	Rise time (m.sec.) <sup>±</sup> s.d.	1/2 fall (m.sec.) <sup>±</sup> s.d.
I Control	25	4.3920 <sub>±</sub> .2017	0.5480 <sub>±</sub> .0583	0.5040 <sub>±</sub> .0538
Treat	7	5.0710 <sub>±</sub> .1603	0.9285 <sub>±</sub> .0948	0.5142 <sub>±</sub> .1212
<u>t</u>		<u>9.4174*</u>	<u>10.9971*</u>	<u>0.2227</u>
II Control	25	0.9800 <sub>±</sub> .0574	0.6240 <sub>±</sub> .0519	1.0760 <sub>±</sub> .1330
Treat	25	1.4160 <sub>±</sub> .1104	0.7720 <sub>±</sub> .0608	1.1080 <sub>±</sub> .1469
<u>t</u>		<u>19.5515*</u>	<u>10.4964*</u>	<u>0.8888</u>
III Control	14	2.1214 <sub>±</sub> .1311	0.9142 <sub>±</sub> .0860	1.2428 <sub>±</sub> .0754
Treat	18	2.9111 <sub>±</sub> .1811	1.2000 <sub>±</sub> .1746	1.3333 <sub>±</sub> .1746
<u>t</u>		<u>14.9281*</u>	<u>6.3937*</u>	<u>2.0246</u>

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\* Statistically significant difference between control and treated responses ( $P < 0.05$ ).

25 fluoride treated e.p.p.'s. In the third experiment, 14 control e.p.p.'s were compared with 18 treated values.

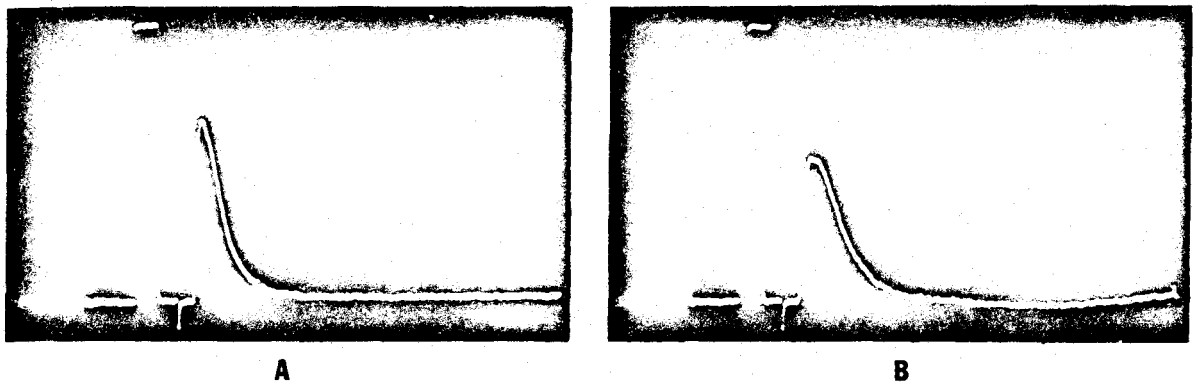
In all three experiments, 8mM NaF significantly increased the amplitude of the e.p.p. in the d-tubocurarine blocked muscle ( $P < 0.05$ ). Rise time also increased significantly in all three experiments. One-half fall, an indication of cholinesterase inhibition, appeared unaffected in our studies.

Figure 9 illustrates the action of 8mM NaF in the absence of d-tubocurarine. In this illustration, muscle movement was prevented by cutting the muscle fibers with a fine scalpel and allowing resting potential to decline above the muscle action potential threshold. Panel A in the figure illustrates a control e.p.p. and panel B the effect of 8mM NaF. As can be seen, perfusion with 8mM NaF led to a significant decline in e.p.p. amplitude and a change in e.p.p. time course. The e.p.p.'s appeared rounded at their peak and declined more slowly than in the control.

Table 12 summarizes the results of an experiment in which time course studies were done. These data demonstrate that 8mM fluoride, in the absence of a d-tubocurarine block, depressed e.p.p. amplitude and increased rise time and 1/2 fall.

Several interpretations of these data are pos-

FIGURE 9



E.p.p.'s recorded in cut-fiber muscle. A=control; B=8mM NaF. Calibration pulse=10mVx1msec; stimulation interval=0.2sec.

TABLE 12

Effect of 8mM NaF on Amplitude and Time Course  
of e.p.p.'s in cut-fiber preparation

<u>Treatment</u>	<u>N</u>	<u>R.P.</u> <u>(mV)</u>	<u>Amplitude</u> <u>(mV)<sup>+</sup>s.e.</u>	<u>Rise Time</u> <u>(msec)<sup>+</sup>s.e.</u>	<u>1/2 fall</u> <u>(msec)<sup>+</sup>s.e.</u>
Control	40	34.7	6.31 $\pm$ .03	0.58 $\pm$ .04	0.92 $\pm$ .03
8mM NaF	40	32.0	5.18 $\pm$ .18*	0.71 $\pm$ .06*	1.40 $\pm$ .04*

---

\* Statistically significant difference between control and treated groups ( $P < 0.05$ ).



sible. It would appear that the depressed e.p.p. could explain the neuromuscular blocking action of fluoride seen in our studies on muscle movement and reported by Blaber (1970). The prolonged 1/2 fall may reflect some degree of cholinesterase inhibition.

If one were to consider "sensitization" as a possible mechanism, one might explain the prolonged 1/2 fall of these e.p.p.'s as due to increased receptor binding by ACh. The depressed amplitude of the e.p.p. may be due to a subsequent competitive antagonism of ACh with itself.

If one considers a presynaptic mechanism of fluoride to explain the results seen, one could envision a change in time course of the release mechanism as accounting for these results. For example, if ACh was released over a longer period of time, the e.p.p. would rise more slowly, not reach the amplitude obtained in the control, and decline slowly.

A third interpretation is that the observed e.p.p.'s resulted from both a pre and postsynaptic action of NaF. For example, fluoride could act at presynaptic sites to depress quantal release and at postsynaptic sites to prolong the action of ACh.

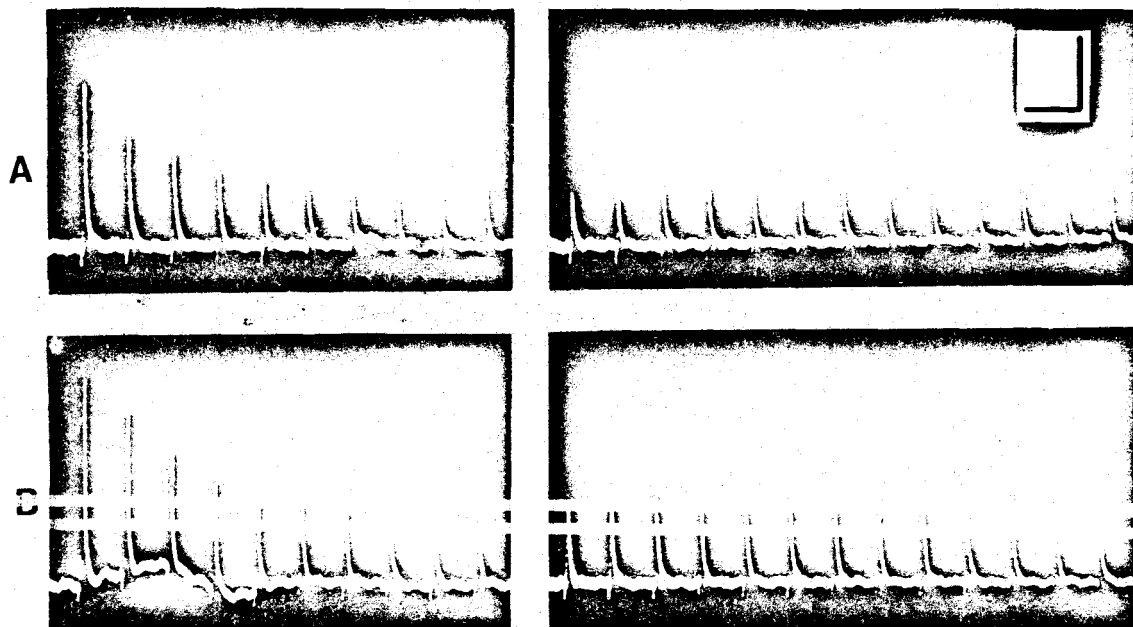
To summarize the data at this point, I have found that in the presence of d-tubocurarine 8mM NaF increases

the amplitude of the e.p.p. but not the amplitude of the m.e.p.p. This suggested to me that NaF may possess a presynaptic action that could result in an elevated quantal content of the e.p.p. These studies with fluoride in the absence of d-tubocurarine showed that this agent increased the amplitude of the m.e.p.p. but depressed and prolonged the e.p.p.

It was decided on the basis of this data on e.p.p.'s and m.e.p.p.'s to compare the action of fluoride in the presence and absence of d-tubocurarine for effects on quantal size and quantal content of the e.p.p. Estimates were also made of readily releasable stores, probability of release, and mobilization of quanta. These determinations, although indirect, could provide additional information that may help to explain the anticurare action of fluoride.

Quantal content of the e.p.p. - Figure 10 illustrates a sample obtained from a single experiment in which the effects of 8mM NaF were investigated for effects on trains of e.p.p.'s in the case of a d-tubocurarine blocked junction. The frequency of stimulation in this case was 25/sec applied at one minute intervals. The control data, indicated under A in the illustration, shows the initial decline in the first

FIGURE 10



E.p.p.'s recorded in d-tubocurarine blocked muscle. Upper two panels (A)=first 10 e.p.p.'s (left) and last 13 e.p.p.'s (right) in a train of 100 in control period. Lower two panels (B)=first 10 e.p.p.'s (left) and last 13 e.p.p.'s in a train of 100 e.p.p.'s 10 minutes after addition of 8mM NaF. Stimulus frequency=25/sec; Calibration=10mVx100msec.

3 or 4 e.p.p.'s (left panel). The right panel shows the last 13 e.p.p.'s occurring in the train. Each train was composed of 100 e.p.p.'s.

Panel B illustrates the same samples of e.p.p.'s taken 10 minutes after perfusion with 8mM NaF. As can be seen in these samples, the amplitude of each e.p.p. in the treated group (panel B) was larger in amplitude than the controls (panel A). This verifies our earlier studies of NaF with regard to d-tubocurarine blocked e.p.p.'s collected at a stimulus frequency of 0.2/sec.

In table 13 are listed the measurements made from a series of 6 experiments. Each value indicates the mean response from the 6 experiments. Statistical comparisons were carried out by paired t-test in which each animal served as his own control. Frequency of stimulation was 25/sec applied at one minute intervals.

In these studies, quantal size increased only slightly between the control and treated groups. In none of the individual experiments were statistically significant changes noted.

Quantal content increased significantly in the presence of 8mM NaF as did readily releasable stores. Fractional release remained unchanged.

It appeared from these results that the action

TABLE 13

Effect of 8mM NaF on transmitter storage and release

	<u>Mean<sup>†</sup>s.e.</u>	<u>N=6</u>
	Control	8mM NaF
Quantal Size (q)	0.0095 <u>±</u> 0.0019	0.0112 <u>±</u> 0.0023
Quantal Content ( $M_0$ )	241.0 <u>±</u> 27.1	386.0 <u>±</u> 57.1*
Available Store (N)	962.0 <u>±</u> 189.8	1452.0 <u>±</u> 282.0*
Fractional Release (P)	0.2505 <u>±</u> 0.0169	0.2876 <u>±</u> 0.0125

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\* Statistically significant difference between control and treated groups ( $P < 0.01$ ). Stimulation frequency = 25/sec.

of NaF in d-tubocurarine blocked muscle was associated with a rise in readily releasable stores and subsequent increase in e.p.p. quantal content. This, it was felt, could explain the anticurare action of NaF.

These studies were repeated at a higher frequency of stimulation (100/sec) to parallel as closely as possible conditions which may reveal the blocking action of NaF exerted with regard to one muscle twitch. Another purpose was to improve the estimate of probability of release and readily releasable stores. This could be accomplished by increasing the stimulus frequency to exceed mobilization. By doing this, a greater number of e.p.p.'s occurred during the early declining phase of a train of e.p.p.'s thus providing more points to estimate probability of release and readily releasable stores.

Table 14 summarizes the results from 6 cats. Added to this table was an estimation of mobilization, measurements of amplitudes of the first e.p.p. in each train, and the mean resting potentials for the 6 experiments. As evidenced in these studies, 8mM NaF increased quantal content significantly. Similar to the data obtained for 25/sec stimulation frequency, releasable stores also increased significantly at a stimulation frequency of 100/sec. Supporting the earlier data at

TABLE 14

Effect of 8mM NaF on neuromuscular transmission in  
normal curare-blocked preparation

	<u>Mean</u> <u>±</u> <u>s.e.</u>	<u>N=6</u>
	Control	8mM NaF
Quantal Size (q)	0.0131 <u>±</u> 0.0055	0.0128 <u>±</u> 0.0018
Quantal Content ( $M_0$ )	216 <u>±</u> 29	346 <u>±</u> 52*
Mobilization (q/m sec)	5.74 <u>±</u> 0.94	7.64 <u>±</u> 0.53*
Available Stores (N)	1042 <u>±</u> 175	1465 <u>±</u> 150*
Probability of Release(P)	0.2261 <u>±</u> 0.0310	0.2318 <u>±</u> 0.0241
Amplitude of first e.p.p. (mV)	2.50 <u>±</u> 0.30	3.78 <u>±</u> 0.48*
Resting Potential (mV)	73.3 <u>±</u> 1.4	73.3 <u>±</u> 1.4

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\* Significant difference ( $P < 0.05$ ) between paired control and treated values, one-tail test. Stimulation frequency= 100/sec.

0.2/sec frequency, e.p.p. amplitudes were significantly increased after perfusion with 8mM NaF for 10 minutes. Mobilization increased in these studies, thus suggesting that the increased readily releasable stores were produced by a stimulating effect of fluoride on mobilization.

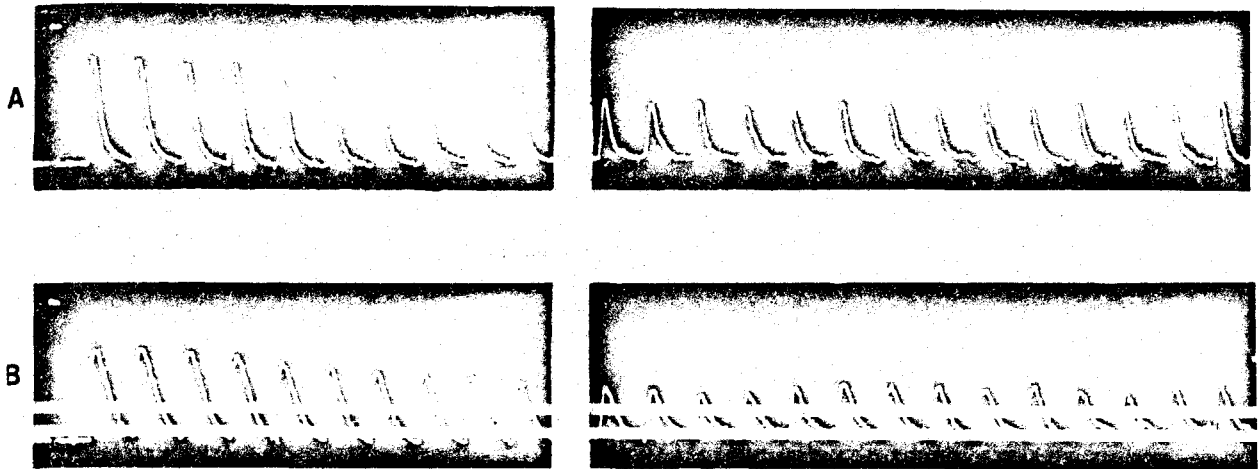
Resting potentials were not altered in any of these studies, although it should be noted that the measurement of resting potential was relatively crude and small changes (less than 2mV) went undetected. Similar to the studies at 25/sec, probability of release and quantal size remained unaffected by administration of 8mM NaF.

Studies of the effect of NaF in the absence of d-tubocurarine were undertaken utilizing the cut-fiber technique. In this case, a series of experiments were undertaken in which stimulation frequency was raised to 200/sec. This was necessary to obtain a sufficient decline in e.p.p. amplitude in order to measure probability of release and readily releasable stores.

Figure 11 illustrates samples of data obtained from a single cut-fiber experiment. The two panels labeled A show components of a train of e.p.p.'s. The left panel shows the first 10 e.p.p.'s and the right panel the last 14 e.p.p.'s in a train of 100 control e.p.p.'s. As can be seen in the left panel, the e.p.p.'s



FIGURE 11



E.p.p.'s recorded in cut-fiber muscle. Upper two panels (A)=first 10 e.p.p.'s (left) and last 14 e.p.p.'s (right) in a train of 100 in control period. Lower two panels (B)=first 10 e.p.p.'s (left) and last 13 e.p.p.'s (right) 10 minutes after addition of 8mM NaF to perfusion. Stimulus frequency=200/sec; Calibration pulse-10mVx1msec.

decline at a much slower rate in the cut-fiber preparation than in the d-tubocurarine blocked muscle (c.f. figure 10).

After treatment with 8mM NaF, the individual e.p.p.'s in the example became depressed and prolonged (panel B). These data parallel the earlier observation on the depressant effect of 8mM NaF seen at a stimulation frequency of 0.2/sec.

Table 15 contains the results of 8 experiments analyzed in a manner identical with that followed in the studies in d-tubocurarine blocked muscle. In these studies, mobilization was significantly depressed by 8mM NaF. Readily releasable stores were also significantly depressed by fluoride, while probability of release increased.

These data suggest that perhaps a component of the neuromuscular blocking action of NaF may be due to a depression of mobilization that is associated in some way with an elevated probability of release. This dual action of fluoride could, on the one hand, decrease filling of the readily releasable pool by depressing mobilization, and, on the other hand, lead to an enhanced depletion of the releasable pool by increasing the probability of release.

Thus, these data could explain the gradual de-

pression of neuromuscular transmission seen in the preparations that were treated with fluoride and subjected to intermittent tetanic stimulation.

TABLE 15

Effect of 8mM NaF on neuromuscular transmission in  
cut-fiber preparation

	<u>Mean<sup>+</sup>s.e.</u>	<u>N=8</u>
	Control	8mM NaF
Quantal Size (q)	0.0876 <sub>±</sub> 0.0252	0.0968 <sub>±</sub> 0.0293
Quantal Content (M <sub>0</sub> )	273 <sub>±</sub> 27	258 <sub>±</sub> 37
Mobilization (q/msec)	16.92 <sub>±</sub> 2.89	14.7212 <sub>±</sub> 2.5974*
Quantal Stores (N)	2441 <sub>±</sub> 437	1929 <sub>±</sub> 428*
Probability of Release(P)	0.1162 <sub>±</sub> 0.0122	0.1443 <sub>±</sub> 0.0199*
Amplitude of first e.p.p. (mV)	8.33 <sub>±</sub> 0.84	7.93 <sub>±</sub> 0.93
Resting Potential (mV)	35.0 <sub>±</sub> 4.3	34.3 <sub>±</sub> 4.8

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\* Significant difference ( $P < 0.05$ ) between paired control and treated values, one-tail test. Stimulation frequency= 200/sec.

## DISCUSSION

Sodium fluoride has enjoyed a long history in the hands of the physician, pharmacist, and chemist. This salt has been sporadically introduced for therapeutic or antiseptic purposes over the last 85 years. Nothnagel and Rossbach (1884) concluded that fluoride was without physiologic or pharmacologic importance. Koplipinski (1886), on the other hand, suggested that fluoride possessed important antiseptic and analgesic properties. Early reports on the toxicology of NaF were numerous. These reports dealt with fluoride in a rather descriptive manner. Results of experimental studies were grouped with other salts and reported in an encyclopedia format (e.g. Rabateau, 1867). Due in part to lack of clear detail about experimental designs, doses, etc., fluoride earned an early reputation as a notorious poison (Rabateau, 1867; Shulz, 1889; Tappeiner, 1890, 1899; Hewelke, 1890; Wieland and Kurtzahn, 1923; Dalla Volta, 1924; Leake, 1928; Costani, 1933; McClure, 1933; Roholm, 1937; and Lidbeck, Hill and Beeman, 1943). Sollman (1957) indicated that NaF is a "general protoplasmic poison, of some toxicological importance and considerable scientific interest". Similar summaries alluding to the toxic properties of

this agent have been stated by Dreisbach (1966). Cutting (1970) cites fluoride toxicity as specifically due to general enzyme inhibition. Goodman and Gilman (1970) summarized all pharmacological activity of fluoride excluding dental properties as "toxic".

Since fluoride has been shown to be effective in preventing dental caries at safe concentrations in drinking water (Hodge, 1956), a sharp increase in the number of investigations of NaF effects on bone, teeth, and metabolism has occurred. A large number of these investigations can be described as toxicity studies. Hodge and Smith (1965) in reviewing the literature developed over 27 years (1934-1964) summarized the mechanism of death in fluoride poisoning as due to the following combination of factors:

- 1) Blockade of normal metabolism in cells.
  - 2) Inhibition of essential enzymes.
  - 3) Cessation of the origin and transmission of nerve impulses.
  - 4) Interference with body functions controlled by  $\text{Ca}^{++}$ , e.g. blood clotting and membrane permeability.
  - 5) Cell damage and necrosis producing massive impairment in the function of vital organs.
  - 6) Terminally there is a shock-like syndrome.
- These toxic signs, although perhaps not directly re-

lated to neuromuscular transmission, may provide some descriptive evidence of an underlying mechanism of action of fluoride at the neuromyal junction.

More relevant to the studies on neuromuscular transmission are the many experiments with fluoride of biochemical, metabolic, physiological, and pharmacological design.

Enolase. - Enolase is any enzyme which functions in the anaerobic metabolism of carbohydrates (Embden-Meyerhof Pathway of Glycolysis). Specifically, 2-phosphoglyceric acid (a metabolite of glucose-6-phosphate) is dehydrated to phosphoenol pyruvic acid by pyruvate kinase yielding 2 moles of ATP. Under anaerobic conditions ketopyruvic acid is reduced to lactic acid. Under aerobic conditions the formation of lactic acid is not required, and pyruvic acid becomes the final product of glycolysis. The pyruvic acid formed serves as a substrate for the formation of acetic acid which in turn enters the citric acid cycle. On the other hand, lactic acid is formed under conditions of muscle exercise and serves as an additional mechanism for the reoxidation of NAD. The enolase reaction is specifically blocked by NaF (Warburg and Christian, 1942). Since  $Mg^{++}$  and phosphate are required for the activation

of enolase, it is believed that the mechanism of action of fluoride occurs through competitive antagonism of  $Mg^{++}$  ions by  $[Mg^{++}][F]^2[PO_4]$ , a complex formed in the presence of fluoride (Warburg and Christian, 1942; and Peters, Shorthouse and Murray, 1964). The inhibition of enolase by fluoride is enhanced by phosphate ions. The effective concentration of fluoride is  $5.0 \times 10^{-4}M$  (45% inhibition).

Succinic dehydrogenase. - This enzyme activates the oxidation of succinic acid to fumaric acid. Succinic dehydrogenase is a ferriflavoprotein associated with cellular mitochondria involved in cellular oxidation-reduction processes. Fluoride at concentrations of  $9.1 \times 10^{-4}M$  was found to inhibit competitively this enzyme (Slater and Bonner, 1952). This inhibition is greatly enhanced by phosphate. The latter effect is not due to the formation of fluorophosphate ( $FPO_3^-$ ) (Slater, 1954) but may be due to fluoroferriphosphate (Massey, 1958).

Malonic acid is a specific inhibitor of succinic dehydrogenase. Generally, inhibition by fluoride results in the accumulation of succinate and alpha-ketoglutarate in tissues (e.g. muscle).



Phosphoglucomutase. - This enzyme activates the formation of glucose-6-phosphate to glucose-1-phosphate in the presence of  $Mg^{++}$  ions as part of the uronic acid pathway in glycolysis. This pathway serves in the formation of glycogen. Concentrations of fluoride at  $1.5 \times 10^{-3}M$  are inhibitory to this enzyme.

Najjar (1948) has studied the effect of fluoride in isolated rabbit muscle. His results were analogous to that found with enolase. In this case fluoride formed fluoromagnesium glucose phosphate which in turn competitively inhibited  $Mg^{++}$ . Phosphate intensified the inhibition.

Adenylate kinase (myokinase). - This enzyme activates the formation of ATP and AMP from ADP. Studies in muscle (Barkulis and Lhninger, 1951; Callaghan and Weber, 1959) have shown that NaF in concentrations of  $2.0 \times 10^{-2}M$  to  $3.0 \times 10^{-2}M$  inhibit myokinase by more than 50%.  $Mg^{++}$  ions are required for activation of the enzyme.

Creatine transphosphorylase. - This reaction yields ATP and creatine from phosphocreatine in the presence of ADP. This reaction provides an important pool of ATP as a source for energy for contraction of muscle cells. Inhibition of creatine phosphokinase by fluoride

occurs at  $1.0 \times 10^{-3} \text{M}$  (21%) and  $6.5 \times 10^{-2} \text{M}$  (100%) (Ennor and Rosenberg, 1954).  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions are required for activation of the enzyme.

Cholinesterase. - This enzyme is responsible for terminating the action of ACh at the neuromuscular junction and elsewhere. The enzyme is inhibited by fluoride in human plasma at  $5.0 \times 10^{-5} \text{M}$  (Harris and Whittaker, 1963-64). In atypical serum cholinesterase from succinylcholine sensitive individuals, fluoride concentrations as high as  $10^{-2}$  to  $10^{-1} \text{M}$  are required to produce significant inhibition.  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and manganese are activators for this reaction. It is of interest to note that similar data is obtained with high concentrations of NaCl ( $> 1.0 \text{M}$ ). Dybing and Loe (1956) have estimated the  $\text{ED}_{50}$  obtained with fluoride at  $10^{-2} \text{M}$  in cholinesterase from plasma, erythrocytes, and brain. Their data is presented in the following table:

Concentration	Activity ( $\mu/\text{CO}_2/30 \text{ min.}$ )		
NaF (mM)	plasma	erythrocyte	brain
.0	45.1	68.1	84.8
1.0	42.4	58.3	75.9
5.0	27.8	60.4	47.2
10.0	20.6	34.7	33.6
50.0	11.8	9.8	12.0
100.0	5.6	2.1	9.2

Heilbronn (1965a) has studied the anticholinesterase action of fluoride extensively. Results of this study indicate that fluoride is a reversible inhibitor of cholinesterase with a  $K_i$  of 0.4 to  $2.8 \times 10^{-3}M$ . Fluoride is a noncompetitive inhibitor of cholinesterase with a relative activity equivalent to that seen with N-methylpyridinium-2-aldoxime methane. It is not clear if fluoride reacts with the anionic site, esteratic site, or both on the acetylcholinesterase molecule.

Glutamine synthetase. - Glutamine synthetase is an enzyme which is responsible for the formation of glutamine, a constituent of tissue and blood in which it functions as a large proportion of a free amino nitrogen pool. In this role glutamine serves as a source of material for protein synthesis. Examples of the use of glutamine include the synthesis of hexoseamine, purines, histidine, and DPN. Glutamine supplies free nitrogen at the kidney level for the neutralization of cations in urine formation thus conserving blood sodium ions. Glutamine also serves to store ammonia produced in deamination processes and thereby avoiding accumulation of metabolic products in tissue and blood. In brain tissue glutamine

ranks fourth amongst the ten amino acids present in highest concentration in the brain (Sourkes, 1962). Glutamine has been related to brain metabolism in a number of ways:

- 1) It is present for the control of oxidative metabolism by the tricarboxylic acid cycle.
- 2) It indirectly influences the utilization of grain glucose.
- 3) Glutamic acid, the precursor of glutamine, also serves as a precursor for  $\gamma$ -aminobutyric acid, a possible synaptic transmitter.

Fluoride at a concentration of  $10^{-3}M$  inhibits glutamine synthetase by 95% (Elliot, 1953). The synthetase reaction is activated by  $Mg^{++}$  ions.

Alcohol dehydrogenase and calcium activated amylase are enzymes also inhibited by fluoride (Sund and Theorell, 1963; Planes and Theorell, 1961; and Whitaker and Tappel, 1962). Alcohol dehydrogenase is inhibited at concentrations of  $4.9 \times 10^{-2}M$  and  $8.8 \times 10^{-2}M$ . Calcium activated alpha-amylase on the other hand is generally inhibited by all salts. The order of the salts bares a general correlation between the inhibitory ability and the size of the hydrated ion (Wiseman, 1970; and Whitaker and Tappel, 1962).

There are some enzymes activated by metal ions

which are not inhibited by fluoride. An example of one of these is nucleoside diphosphokinase, an enzyme requiring  $Mg^{++}$ . Concentrations up to  $10^{-2}M$  fluoride were unable to inhibit this enzyme (Kirkland and Turner, 1959). The reason why fluoride combines with the metal ion cofactor in some cases but not in others may be related to the affinity of the fluoro-complex for the enzyme compared to that of the cofactor (Wiseman, 1970).

Liver esterase. - The inhibition was initially reported by Hofstee (1954). This author reported that  $5.0 \times 10^{-3}M$  inhibited this enzyme about 50%. McGaughey and Stowell (1964) reported a 50% inhibition at  $6.0 \times 10^{-7}M$  sodium fluoride. The study of Hofstee (1954) was carried out at a pH of 8.0, whereas McGaughey and Stowell (1964) studied the effects of fluoride at a pH of 3.0. The functional role of liver esterase is not known.

Adenylic deaminase. - The function of adenylic deaminase is not clear (West, Todd, Mason and Van Bruggen, 1966). It has been suggested that it prevents the escape of AMP from intracellular to extracellular space. It may be of importance in decreasing acidity due to accumulation of lactic acid in pro-

longed muscular activity by supplying  $\text{NH}_3$ . Adenylic deaminase is present in muscle sarcoplasm and has been associated with the myofibril. Concentrations of sodium fluoride of  $3.0 \times 10^{-2}\text{M}$  inhibit adenylic deaminase about 88%.

Phosphoglyceromutase. - Phosphoglyceromutase functions in glycolysis, a process of carbohydrate metabolism characteristic of animal cells. The general reaction pathway (Embden-Meyerhof Pathway) provides pyruvic and lactic acid for oxidation to  $\text{CO}_2$  and water in the tricarboxylic acid cycle. This process is the source of most of the utilizable energy (ATP) derived from carbohydrate metabolism. Phosphoglyceromutase specifically functions to form 2-phosphoglyceric acid from 3-phosphoglyceric acid. Cowgill and Pizer (1956) have demonstrated that  $1.0 \times 10^{-2}\text{M}$  to  $5.0 \times 10^{-3}\text{M}$  sodium fluoride inhibited phosphoglyceromutase 100% and 90% respectively. Pizer (1963) suggests from their studies that enzyme, substrate, and fluoride form an irreversibly inactivated unit.

Cellular metabolism. - There are three metabolic pathways for production of cellular energy - fatty acid oxidation, Embden-Meyerhof pathway, and

hexose monophosphate shunt. The cell derives its energy by trapping part of the energy released at the oxidative steps in these pathways as adenosine triphosphate (ATP).

Fluoride is known to inhibit the Embden-Meyerhof pathway. In fact, fluoride was an important tool utilized in describing this particular sequence of reactions. Gemmill (1939), Dorfman (1942-43), and Borei (1945) have reviewed details of the effects of fluoride on this pathway. In general, concentrations of  $1.0 \times 10^{-2} \text{M}$  to  $2.0 \times 10^{-2} \text{M}$  NaF inhibit glucose uptake and lactate formation in frog heart and rat heart (Mann and Lutwak-Mann, 1948; and Matthews, 1970). Sbarra and Shirley (1963) found glycogen utilization and lactate production depressed in leucocytes. In rat intestines, Detheridge, Matthews and Smyth (1966) reported inhibition of glucose uptake and lactate formation with  $5.0 \times 10^{-3} \text{M}$  NaF. Matthews (1970) has suggested that the most important inhibition is the effect of fluoride on enolase. McKinney and Martin (1956) have demonstrated a biphasic action of fluoride on glycolysis. Concentrations of  $1.0 \times 10^{-3} \text{M}$  NaF stimulated lactate production in intact cells. At  $1.0 \times 10^{-2} \text{M}$  fluoride inhibited lactate production.

The hexose monophosphate shunt (HMP) appears also to be blocked by fluoride, since this pathway

is only the first part of the EMP before enolase (Matthews, 1970).

Pyruvate metabolism. - Miller and Olson (1954) demonstrated that  $1.0 \times 10^{-3} \text{M}$  NaF inhibited pyruvate utilization on avian heart by 70%. These results demonstrated that pyruvate metabolism is inhibited when lactate was not. In contrast, Rice and Berman (1961) showed a stimulation of pyruvate metabolism in electrically stimulated strips of ventricle at about  $4.0 \times 10^{-3} \text{M}$  NaF. Hashimoto (1961) demonstrated a marked increase in pyruvate in isolated frog hearts at  $1.0 \times 10^{-2} \text{M}$  to  $2.0 \times 10^{-2} \text{M}$  NaF. At this concentration of fluoride, glucose uptake was inhibited. These data suggest rather high concentrations of fluoride are required for significant stimulation of pyruvate metabolism.

Fatty acid oxidation. - Cheldelin and Beinert (1952) showed that  $3.3 \times 10^{-2} \text{M}$  fluoride inhibits butyrate metabolism. This inhibition resulted in a reduction of acetoacetate.

Aisenberg and Potter (1955) showed that in fact acetate activation was inhibited. Inhibition of the conversion of acetate to acetylcoenzyme-A was inhibited



by concentrations of fluoride from  $1.0 \times 10^{-4} \text{M}$  to  $3.0 \times 10^{-3} \text{M}$ .

Respiration. - Miller and Olson (1954) have shown inhibition of cellular oxygen uptake. Concentrations of  $1.0 \times 10^{-2} \text{M}$  to  $2.0 \times 10^{-2} \text{M}$  are required before significant inhibition occurs. In contrast Siekevitz and Potter (1953) have shown that at  $1.0 \times 10^{-3} \text{M}$  NaF stimulates oxygen uptake in some tumor tissues. Hashimoto (1961) showed a progressive increase in oxygen uptake at  $1.0 \times 10^{-3} \text{M}$  in isolated frog hearts.

Spermatozoa. - Some investigations have been carried out on the effects of NaF on cellular movements of various kinds. Mann and Lutwak-Mann (1948) demonstrated that  $2.0 \times 10^{-2} \text{M}$  NaF immobilized spermatazoa. They correlated this response with the inability of fluoride poisoned permatozoa to metabolize fructose. Lindahl and Wedin (1963) correlated this response with the number of "units" (i.e. receptors) in each cell that had to be inhibited to immobilize spermatozoa. From their data it is seen that  $1.2 \times 10^{-2} \text{M}$  NaF immobilized 80% of spermatozoa in about 8 minutes. The rate of immobilization increased with increased fluoride concentration in a linear fashion.

Leucocytes. - Phagocytosis in leucocytes is inhibited at concentrations of  $2.0 \times 10^{-2} \text{M}$  NaF (Sbarra and Karnovsky, 1959). Sbarra and Shirley (1963) demonstrated that fluoride inhibited phagocytosis could be reversed by pyruvate, thereby suggesting that this action is due to inhibition of glycolysis. In apparent contradiction of this assumption, Selvaraj and Sbarra (1966a) have suggested that pyruvate antagonism of fluoride does not act through glycolytic pathways but merely as an alternative energy source.

Heart muscle. - Contractility in heart muscle has been extensively investigated by numerous workers. In general, fluoride stimulates heart muscle (Bennet and Chenoweth, 1952). These authors compared the effect of  $5.0 \times 10^{-3} \text{M}$  NaF with  $1.5 \times 10^{-4} \text{M}$  ouabain on the isolated cat papillary muscle. Anoxia and high concentrations of glucose antagonized this effect for both agents. Fluoride is capable of antagonizing ouabain.  $1.0 \times 10^{-4} \text{M}$  butyrate augments the response of the muscle to fluoride but not to ouabain. Blocked muscle is antagonized by ouabain but not by fluoride in  $\text{Mg}^{++}$  free Ringer's solution. Thiopental, thyroxine, and thiocyanate all block the action of ouabain but not the stimulating action of fluoride.

Loewi (1955) studied the effects of 2.5 to 5.0x 10<sup>-3</sup>M NaF on the isolated frog heart. Loewi demonstrated that fluoride restored the amplitude of contraction of failing preparations caused either by long perfusion, low Ca<sup>++</sup>, or high K<sup>+</sup> ion concentration. Oleic acid also was found to have similar effects. In addition both fluoride and oleic acid form insoluble salts with Ca<sup>++</sup>. Although the results of his study could be explained on the basis of precipitated Ca<sup>++</sup>, Loewi found fluoride to be ineffective in the absence of Ca<sup>++</sup>. He suggested that the prime effect of fluoride lies in its ability to form a non-diffusible complex with the cell surface. This complex once formed restores the sensitivity of the membrane.

Covin and Berman (1959) studied the effects of NaF on isolated rat ventricles. In their studies fluoride increased the force of contraction in a substrate free solution. Fluoride also increased the force of contraction in the presence of glucose and in the presence of pyruvate. In the presence of acetate, fluoride had a negative action. The results of their studies indicated fluoride may act through two different pathways:

- 1) Fluoride stimulates substrate oxidation and makes more energy available.

2) Fluoride may promote a more efficient utilization of energy.

These authors demonstrated that oxygen uptake was not increased by fluoride, hence it would appear that stimulation of substrate oxidation would not have taken place during increased muscle contraction. In interpreting their results, Covin and Berman suggest that the source of energy for increased ventricular contraction is fatty acid. This endogenous substance competes with acetate for essential cofactors and, hence, suppression of fluoride action is obtained by administration of acetate. This effect of fluoride is markedly different from effects seen with ouabain (Majeski and Berman, 1965).

Hashimoto (1961) studied the effect of NaF on isolated frog hearts.  $1.0 \times 10^{-2} \text{M}$  to  $2.0 \times 10^{-2} \text{M}$  fluoride reduced the output and shortened the relaxation period at a time when aerobic glycolysis was inhibited. Pyruvate production and oxygen uptake were increased. He suggested that at low concentrations fluoride stimulates the heart by reacting with  $\text{Ca}^{++}$  at the cell surface. At high concentrations fluoride affects cellular metabolism.

Katzung et al. (1957) demonstrated that fluoride effects on the heart were independent of stimulation

frequency. His studies pointed out that fluoride action differed from that of several other metabolic inhibitors. Cyanide, DPN, and iodoacetate all depressed contractability, while fluoride potentiated the frequency responses (treppe). Matthews (1970) suggests that the effects of fluoride on the heart observed by Katzung may be due to an action on energy metabolism.

Roy (1963) studied the relationship of metabolically dependent movements of  $\text{Na}^+$  and  $\text{K}^+$  on maintenance of contractility. He investigated the action of NaF on arrhythmia induced by ACh in isolated rabbit atria.  $2.5 \times 10^{-4}\text{M}$  NaF was effective in antagonizing ACh induced arrhythmia in about 20 minutes. He concluded that the most probable site of action of fluoride was on the system supplying energy for active ion transport.

#### Smooth muscle and rectus abdominus muscle. -

Vallette and Ozan (1964) investigated the ability of fluoride to modify the response of skeletal muscle (frog rectus abdominus) and smooth muscle (guinea pig ileum) to depolarization contracture produced by high concentrations of potassium chloride.  $4.0 \times 10^{-4}\text{M}$  NaF markedly augmented the contracture in both rectus abdominus and ileum. These authors suggest that the

potentiating effect of fluoride is due to "enzyme inhibition".

Caruso et al. (1970) reported on effects of NaF on dog gut in vivo. Intravenous doses of 1-25mg/Kg to dogs increased gastrointestinal tone without affecting motility. This effect was abolished by atropine and also by ganglion blockers (pentolinium and hexamethonium). Bilateral vagotomy similarly blocked the increased gut tone due to fluoride administrations. These investigators also studied the effects of NaF on in vitro isolated dog gut preparations. Increased tone and activity were observed with  $7.1 \times 10^{-4} \text{M}$  to  $5.7 \times 10^{-3} \text{M}$  NaF. The effects were largely blocked by atropine or ganglion blocking agents. In another series of experiments, Caruso et al. (1970) used morphine sulfate to suppress ACh release from post ganglionic parasympathetic nerve elements in gut. Morphine like atropine and ganglion blocking agents were found to reduce the excitatory effect of NaF. In other studies Caruso investigated the effect of NaF on dog gut segments which were refrigerated for three to five days. This preparation responds only to direct smooth muscle stimulants. In his studies he was unable to observe the excitatory effect of NaF seen in unrefrigerated gut segments. The refrigeration procedure is reported

to result in ganglion degeneration, leaving essentially a tissue devoid of neural elements. Thus, the overall data suggests that neuronal elements are required for demonstration of the excitatory properties of NaF.

Effect of sodium fluoride on blood pressure. -

The hypotensive effects of NaF were first reported in dogs by Greenwood et al. (1934, 1938). He demonstrated that intravenous doses of 23mg/Kg decreased blood pressure by about 20%. Leone et al. (1956) observed a decrease blood pressure and heart rate and increased respiratory rate at doses of 20-30mg/Kg. ECG irregularities occurred at 31mg/Kg. Bishop, Richardson, and Muhler (1954) reported prolonged peripheral dilation as indicated by increased femoral arterial blood flow after intra-atrial injection of NaF. Caruso (1961) observed decreased heart rate and blood pressure in anesthetized dogs with intravenous doses of 30mg/Kg NaF.

Caruso et al. (1970) has recently reported the results of an investigation on blood pressure effects of NaF. In these studies he observed a marked fall in diastolic pressure reaching a peak 5 minutes after intravenous administration of 3% solution of NaF. Systolic pressure was unchanged. Heart rate decreased and res-

piration increased at the time of the blood pressure change. The hypotensive response to NaF was not altered after cervical vagotomy or spinal section at C-1 or C-3.

In further studies the effect of NaF on the blood pressure responses to intravenous epinephrine, nor-epinephrine, ACh, and histamine were investigated by Caruso et al. (1970). Fluoride failed to influence the blood pressure responses elicited by these compounds. In addition, experimental data demonstrated that tri-pelennamine and atropine did not alter the NaF response. Standard alpha-adrenergic or beta-adrenergic blocking agents failed to affect the fluoride induced hypotension.

Surprising results were obtained when Caruso investigated the effects of ganglion blockade on the NaF induced hypotension. It was observed that instead of the expected hypotensive response to fluoride administration, in the presence of ganglion blockade, a marked hypertension was evoked (Caruso and Maynard, 1963). This response is followed by a sustained hypotension. Similar to the blood pressure response, heart rate increased instead of decreased in the presence of ganglion blockers (e.g. pentolinium). The pressor response can be evoked by fluoride at lower doses than what is required for the depressor response. Experiments were carried out in which fluoride was administered to ad-



renalectomized, adrenal-demedullated, adrenal-denervated, or sham-adrenalectomized animals. All of these procedures except for sham operation reduced the magnitude of the increased blood pressure response to fluoride. The ionotropic and chronotropic effects of fluoride on the heart were not affected by the operative procedures.

Shipley and Wilson (1951) investigated the effects of fluoride on femoral arterial blood flow. In the presence of ganglion blockade, peripheral resistance increased instead of decreased. Spinal section at C-1 or C-2 and bilateral vagotomy failed to alleviate the fluoride induced pressor response suggesting a lack of central involvement in the pressor response.

In other studies by Caruso et al. (1970), using tolazoline or phentolamine as alpha-blocking agents, he was able to block the fluoride induced pressor response with little effect on the positive ionotropic or chronotropic effect on the heart. Dichloroisoproterenol on the other hand blocked the positive ionotropic and chronotropic effects of fluoride.

Ganglion blockade appears to unmask the hypertensive and cardiac stimulatory effects of fluoride in vivo. Caruso et al. (1970) suggested this unmasking effect is due to abolishment of a ganglion reflex path-

way affected by ganglion blockade. Thus, the underlying stimulatory response to fluoride is revealed. In an attempt to investigate this point, he found that:

1) Denervation of carotid and aortic sinuses did not unmask the fluoride activated pressor response.

2) Similar negative data were obtained after cervical, thoracic, lumbar, and sacral root sections.

3) Close arterial administration of fluoride in the region of the carotid sinus did not elicit a pressor response.

Isolated heart. - Effect of fluorides on isolated heart preparations have previously been mentioned. It is important, however, to point out that Caruso et al. (1970) also studied the effect of fluoride on the positive inotropic and chronotropic responses obtained in the preparation. To test whether atrial stimulation was specific for fluoride, sodium chloride, bromide and iodide were also tested. These ions were without a positive chronotropic effect. In other studies DCI was shown to block the effects of fluoride on the isolated heart suggesting that fluoride effects may be mediated via adrenergic mechanisms. Studies in isolated atria from reserpenized dogs demonstrated the stimulatory effect of fluoride was still present.

Fluoride was able to restore spontaneous rhythm in isolated atrial preparations from reserpenized dogs.

The inotropic effect was not evident in these studies however, it has already been noted that the fluoride positive inotropic effect occurs only in stressed preparations (Bennet and Chenoweth, 1952; Covin and Berman, 1959; and Rice and Berman, 1961).

The paradoxical effects obtained with fluoride on the heart and blood pressure do not lend themselves to a "classical" interpretation. To summarize:

- 1) In the normal intact animal blood pressure is decreased and heart rate slowed by fluoride administration. There is evidence of a direct smooth muscle peripheral vasodilation. This response is not blocked by atropine or tripelenamine suggesting neither ACh nor histamine mediate the response. Spinal section and bilateral cervical vagotomy failed to influence the hypotension. Responses to epinephrine, norepinephrine, and ACh were not modified by fluoride. Alpha and beta adrenergic blockers similarly were without effect.

- 2) After administration of ganglion blockers, a pressor response and cardiac stimulatory response to fluoride is unmasked. Tolazoline, an alpha-adrenergic blocker, antagonized the pressor response, while DCI, a beta-adrenergic receptor blocker, depressed the posi-

tive chronotropic and inotropic effects of fluoride on the heart. The pressor response is relieved by adrenalectomy, but the cardiac stimulatory effects are not.

3) The stimulatory effects of NaF on the isolated dog atria were blocked by DCI. Paradoxically, fluoride induced stimulation is still evident in chronically reserpinized dogs.

#### Effects of sodium fluoride on respiration. -

NaF has been found to stimulate respiratory rate (Greenwood et al., 1934, 1935, 1938; Leone, et al., 1956; Lidbeck, et al., 1943; Roholm, 1937; and Caruso et al., 1970).

The underlying mechanism of fluoride action on respiration appears to be due to its inverse relationship to a decreased blood pressure (Caruso et al., 1970). Evidence for this interpretation lies in the fact that part of the respiratory response is mediated through the vagus. Carotid sinus denervation and vagal section almost completely abolish a fluoride evoked increase in respiratory rate.

Paradoxically, the terminal event in acute fluoride poisoning appears to be respiratory paralysis.

Experiments have been carried out to study the mechanism of respiratory arrest. Simultaneous measure-

ment of blood pressure, respiration, and the force of contraction of the gastrocnemius muscle of a dog were measured (Caruso et al., 1970). In these studies, NaF initially increased the force of contraction then depressed it. At the time of respiratory arrest, indirect stimulation via the sciatic nerve failed to elicit a contraction yet direct stimulation of the muscle produced a response. The author suggested failure of neuromuscular transmission was the cause of respiratory arrest. In additional studies, he reported that fluoride did not restore neuromuscular paralysis induced by decamethonium but antagonized a paralysis due to d-tubocurarine.

It is concluded from the present studies with fluoride that this agent produces several effects on neuromuscular transmission. Of the parameters measured, it appears that the anticurare action is probably mediated at a presynaptic site. Stimulation of this site by fluoride leads to increased mobilization of ACh quanta, increased readily releasable stores of ACh, and a subsequent increase in quantal content of the e.p.p. The anticurare action is realized by increasing the amplitude of the e.p.p. to a level sufficient to elicit a muscle action potential.

The thesis does not support the hypothesis that the anticurare action of this agent to its sensitizing

action. Although this action was observed on m.e.p.p.'s, these effects were not evident in the presence of d-tubocurarine.

An unusual depressant affect of fluoride on the e.p.p. was observed in the absence of d-tubocurarine. No explanation as to why, in the absence of d-tubocurarine, fluoride increased the amplitude of the m.e.p.p. but decreased the amplitude of the e.p.p. It may be that the cut-fiber procedure for measuring e.p.p.'s exposes fluoride to sites not available in the normal muscle preparation used to measure m.e.p.p.'s.

## SUMMARY AND CONCLUSIONS

Upon reviewing the very large volume of literature on fluoride, it becomes apparant that the information reported seems to fall into four broad categories. A great deal of the data deals with the toxicity of fluoride, another group deals with the biochemical effects, another with metabolism, and still another with its effect on excitable systems.

In constructing a theory on the mechanism through which fluoride antagonizes d-tubocurarine blockade, it is necessary to utilize, to a certain extent, those systems which are affected by fluoride that may function in some way in neuromuscular transmission.

The physiological consequences of inhibiting various enzymes is a difficult problem to evaluate. As one can see, there are a sufficient number of enzymes inhibited by fluoride to drastically reduce energy producing products from a wide variety of substrates. It is important to point out that there is some question whether some of the actions of fluoride are obtained *in vivo*. Almost all of the biochemical tests with fluoride were carried out after homogenizing the tissue and thus allowing access to systems which fluoride may be unable to reach *in vivo*.

If enolase were inhibited in vivo by fluoride, it could lead to a reduction in glycogenolysis. When the breakdown of muscle glycogen has been prevented, the energy required for the regeneration of high energy phosphate bonds (ATP) is diminished. When poisoned in this manner, a muscle may contract for a period of time then eventually fail because of the depletion of this energy reserve. This result, of course, does not explain the anticurare action of fluoride but may be a component in the gradual neuromuscular block which develops after prolonged exposure to this agent.

Inhibition of succinic dehydrogenase would lead to the accumulation of succinate and alpha-ketoglutarate in tissues and also depression of cellular oxidation-reduction processes. I do not know how to relate the anticurare action of fluoride to this action.

Inhibition of phosphoglucomutase, myokinase, and creatine transphosphorylase, like inhibition of enolase, would all lead to reduction of high energy phosphate and thus limit the ability of the muscle to continuously respond to stimulation over a prolonged period of time.

The inhibition of acetylcholinesterase by fluoride, on the other hand, should facilitate neuromuscular transmission by prolonging the action of acetylcholine



at the end plate. Thus, in the presence of d-tubocurarine, one would expect an increase in the amplitude of the end plate potential and some prolongation of its time course. Similar results would be expected when measuring m.e.p.p.'s.

In the presence of d-tubocurarine, we observed a significant increase in e.p.p. amplitude without a prolongation in time course. Thus, it is possible that this effect may be due to a degree of cholinesterase inhibition that was sufficient to increase e.p.p. amplitude but not prolong its time course. In contrast to this, when the amplitude of the m.e.p.p. was depressed with d-tubocurarine, fluoride was not able to antagonize the depression. This effect is not consistent with what is theoretically expected. If acetylcholinesterase was inhibited, this should have resulted in a reversal of the d-tubocurarine action on the m.e.p.p. amplitude.

When considering the combined effects of fluoride on e.p.p.'s and m.e.p.p.'s, it is tempting to suggest that, since the amplitude of the m.e.p.p. is not increased in the presence of d-tubocurarine while the e.p.p. is increased, the anticurare action of fluoride is due to an action on the nerve terminal. This action results in an increased quantal content.

The other enzymes reviewed do not appear to contribute to explaining some of the pharmacological effects observed with fluoride in our studies. Inhibition of adenylic deaminase like phosphoglyceromutase seems to lead to weakening of muscle contraction more than an explanation of an antitoxic action.

One point should, however, be made about inhibition of all of these enzymes. If any of them are eventually shown to indirectly influence transmitter mobilization or release, they could, under certain circumstances, increase quantal release from the nerve terminal. For example, if one of the high energy phosphate producing enzymes were shown to control the sodium pump in the nerve terminal, it is possible that by inhibiting this system the number of quanta released by each impulse would increase. Evidence for this view has been presented by Blaber(1970) who suggested the depolarization of the nerve terminal leads to transmitter mobilization. Alteration of the time course of the nerve action potential may also lead to increased quantal release.

Another mechanism could be related to the Mg binding action of fluoride. For example, if there was a metabolically active system present in the nerve terminal controlling the gradient between the relatively

small releasable pool of transmitter and very large less readily releasable transmitter store, then inhibition of the controlling enzyme or binding of its activating metal ion by fluoride could lead to an increased readily releasable pool. This in turn could result in increased quantal content of the e.p.p. if probability of release remained unchanged or increased.

The stimulatory effect of fluoride on heart muscle is interesting. Generally speaking this action is similar to actions seen with catecholamines or perhaps with theophylline. The existing data indicates this action is different from digitalis but similar to that seen with oleic acid. Thus, it appears fluoride may have an unusual action on the heart muscle membrane. Loewi has suggested that fluoride forms a complex with Ca that binds to the cell surface thus increasing cell membrane sensitivity. This does not relate well to our observations of the effect of fluoride on quantal content of the e.p.p. In the case of the d-tubocurarine blocked muscle, a reduction in free Ca would in effect decrease the quantal content of the e.p.p. This may, however, still occur since in our cut-fiber preparations we observed a reduction in e.p.p. quantal content. Similarly, one could speculate that the depression of e.p.p. amplitude and prolongation of its time course

seen in our cut-fiber preparation reflect alterations in end plate receptor sensitivity. There is also the possibility that the change in e.p.p. time course could be due to inhibition of acetylcholinesterase.

Of some interest is the possibility that fluoride may stimulate alpha or beta receptors present in the neuromuscular junction. Another possibility is that fluoride at certain concentrations could stimulate the formation of cyclic 3'5'AMP. The only data supporting these concepts are indirect. As previously mentioned it has been shown that fluoride stimulates beta receptors in the heart that are blocked by propranolol, and, in addition, it has been demonstrated by Sutherland and Rall (1960) that fluoride maximally stimulates the formation of 3'5' cyclic AMP. These concepts are not well enough understood as yet to be included as an explanation of an underlying mechanism controlling quantal content of the e.p.p.

Another result of our studies with fluoride demonstrate the long held view of many pharmacologists that mechanisms of drug action differ from one species to the other.

Studies of Koketsu in the frog demonstrated that the anticurare action of fluoride is due to sensitization of the end plate region. In our studies we were

unable to attribute the sensitizing action of fluoride to an antitubocurarine effect. We have concluded from the data that the site of action of fluoride in mammalian tenuissimus muscle is presynaptic. The differences between the frog and cat muscle preparations are not clear. It is possible that there is a different distribution of fluoride sensitive receptors. It should be noted that the frog preparation appears much more sensitive to the post synaptic actions of fluoride in that the increased m.e.p.p. amplitudes were much more marked in the frog than in the cat. In addition, this sensitization of the end plate occurred at a much lower dose in the frog than in the cat.

In analyzing the results of my studies with fluoride in cat tenuissimus muscle, the following findings were noted:

- 1) A postsynaptic sensitization phenomena was demonstrated on the basis of an increased amplitude of the m.e.p.p. resulting from treatment with fluoride. This action of fluoride appears to result in antagonism of d-tubocurarine in isolated frog muscle but not in cat tenuissimus. In the studies presented in this dissertation, it was found that NaF was unable to reverse the blocking action of d-tubocurarine on the m.e.p.p. amplitude. In addition, m.e.p.p.'s which were

increased in amplitude by prior exposure to fluoride were also depressed by subsequent treatment with d-tubocurarine. Two speculations are possible from these data. First, it appears that, if fluoride were a functional acetylcholinesterase inhibitor, it should have reversed the post synaptic action of d-tubocurarine by prolonging the action of ACh at the end plate. This did not occur. Thus, I am unable to relate the anticholinergic action of fluoride to its well established anticholinesterase action. One can speculate that the increased m.e.p.p. amplitude produced by fluoride treatment was not the result of acetylcholinesterase inhibition, since no interaction between these two types of responses were obtained in the presence of d-tubocurarine. Investigations of the effects of fluoride on m.e.p.p. time course could provide additional evidence on this point.

The second point evident from my studies on m.e.p.p. amplitude are based on the assumption that the increased m.e.p.p. amplitude was due to some other mechanism than acetylcholinesterase inhibition. The term "sensitization" used in this context seems to suffer from a lack of clarity as to its site and mechanism. With respect to the end plate region, "sensitization" can be defined as an increased amplitude of the m.e.p.p.

This response could be due to several mechanisms. For example, if the number of nicotinic receptors available in the end plate were to increase, one could theoretically obtain an increased m.e.p.p. amplitude by virtue of more efficient utilization of transmitter. Another definition that may explain sensitization is the interaction of ACh with the receptor. Fluoride may in some way improve the receptor configuration and thus allow ACh to combine with the receptor more efficiently. Under these conditions of improved receptor binding, the association and dissociation times may change and subsequently a greater degree of depolarization at the end plate could be obtained.

Any of these mechanisms could explain the action of fluoride on the m.e.p.p. It must be emphasized however that in the presence of d-tubocurarine m.e.p.p. amplitude changes due to fluoride actions are abolished. Therefore, the evidence presented suggests that the fluoride induced increase in m.e.p.p. amplitude does not play a role in antagonizing d-tubocurarine.

The importance of these findings are related in two ways to our studies of end plate potentials. 1) If the e.p.p. increases in size while the m.e.p.p. remains the same amplitude (or smaller), then the number of quanta in the e.p.p. must have increased. If this

occurred, then fluoride may in fact exert its anti-curare action at the nerve terminal by increasing quantal release of transmitter and not post synaptically at the end plate region. 2) The indirect measurement of quantal content is made by measuring the amplitudes of a train of e.p.p.'s and by certain statistical assumptions leading to an estimation of the quantal components of the e.p.p. If fluoride were to alter the m.e.p.p. size in the presence of d-tubocurarine, then the analysis of the presynaptic action of fluoride would be in doubt, since an estimate m.e.p.p. amplitude is fundamental to all subsequent calculations.

Since the postsynaptic actions of fluoride are blocked out by d-tubocurarine, I believe the analysis of effects of fluoride on e.p.p.'s is valid, and the site of action of fluoride with respect to its anticurare action is in fact presynaptic.

3) The presynaptic action of fluoride was evidenced by the fact that, in the presence of d-tubocurarine, the amplitude of the e.p.p. is increased under conditions in which the m.e.p.p. does not increase in amplitude. Analysis of trains of e.p.p.'s demonstrated that quantal content of the e.p.p. increased progressively up to the time when muscle movement was restored. Quantal size, an indirect estimate of the m.e.p.p.



amplitude, remained unchanged during the treatment with fluoride thus supporting our direct measurements of m.e.p.p.'s. Analysis of individual e.p.p.'s demonstrated that in the presence of d-tubocurarine the amplitude of the e.p.p. increased, while 1/2 fall remained unchanged. If 1/2 fall were to have increased, one could suspect that fluoride actions on the e.p.p. included cholinesterase inhibition. Since it was shown that the latter effect did not occur, it is suggested that the anticurare action of fluoride is associated with an elevated release of ACh from the nerve terminal.

Analysis of trains of e.p.p.'s demonstrated that in the presence of d-tubocurarine probability of release did not increase, while mobilization of transmitter and the size of the readily releasable pool of transmitter did increase. These data suggest that fluoride exerted its anticurare action by increasing the pool of releasable transmitter. One can visualize, therefore, that in the presence of fluoride, when the nerve action potential invades the nerve terminal, it activates release from a larger pool of transmitter. Since the probability of release remained unchanged, there was a corresponding increase in the number of quanta released. Since the ACh content of a quanta is very high ( $10^4$ - $10^5$  molecules), one would expect that

this elevated release would be sufficient to antagonize the postsynaptic effects of d-tubocurarine.

Interestingly, these presynaptic effects of fluoride are evident only in the presence of d-tubocurarine. When fluoride was studied in the absence of d-tubocurarine in the cut-fiber preparation, different effects were seen. Analysis of individual e.p.p.'s showed that the time course of the e.p.p. was flattened and prolonged. Thus, an e.p.p. of smaller amplitude and longer time course is produced in the presence of fluoride.

Two explanations of these results are possible:

1) The e.p.p. was prolonged by a postsynaptic effect of fluoride. If we maintain that sensitization is associated with an increased binding of transmitter to end plate receptors, then the prolonged 1/2 fall obtained in the absence of d-tubocurarine would be expected. Similarly, inhibition of acetylcholinesterase could explain this result. The smaller amplitude of the e.p.p., however, is not explained by either of these two mechanisms. 2) If the e.p.p. was prolonged by a presynaptic action of fluoride, an interesting explanation of the results evolve. For example, if transmitter release time were increased by fluoride under conditions where the probability of release remained unchanged, then there would be a prolongation

of the e.p.p. observed. If, in addition, the quantal content remained about the same in the presence of fluoride, one would expect the e.p.p. to be of smaller amplitude and somewhat prolonged. This is in fact what was observed in e.p.p.'s obtained from the cut-fiber preparation.

When trains of e.p.p.'s were analyzed in the cut-fiber preparation, it was observed that quantal content and readily releasable stores were slightly depressed. This may well be an artifact in that, under these conditions (i.e. in the absence of d-tubocurarine), quantal size was increased by fluoride. The effects of fluoride on probability of release also were not consistent with what is seen in d-tubocurarine blocked preparations in that in the cut-fiber preparation probability of release increased slightly. There is no clear explanation for this result.

In conclusion, it is felt that these studies with fluoride in cat tenuissimus muscle resulted in the following findings:

- 1) A postsynaptic sensitization phenomena was demonstrated on the basis of an increased amplitude of the m.e.p.p. resulting from treatment with fluoride.

- 2) Evidence for a presynaptic site of action of fluoride was presented.

3) The anticurare action of fluoride is realized by increasing the amplitude of the e.p.p. under conditions in which the m.e.p.p. does not increase in amplitude.

4) The increased e.p.p. amplitude is due to a greater number of quanta being released from the nerve terminal.

5) Fluoride produced an increase in the number of quanta in the readily releasable pool by increasing mobilization of quanta from another transmitter source.

6) Since the probability of release did not change, the number of quanta released increased by virtue of the increased size of the readily releasable pool of quanta.

7) It was pointed out that, although fluoride is anticurare in both the frog and mammalian, there appeared to be differences in its mechanism of action.

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## APPROVAL SHEET

The dissertation submitted by Robert S. Jacobs has been read and approved by a committee composed of D.W.Calhoun, M.S., A.H.Friedman, Ph.D., J.I.Hubbard, Ph.D., S.Nishi, M.D., Ph.D. and A.G.Karczmar, M.D., Ph.D. (Chairman).

The final copies of the dissertation have been examined by the chairman of the examining committee and the signature verifies that all necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is, therefore, accepted in partial fulfillment of the requirement for the Degree of Doctor of Philosophy.

5/24/1971  
Date

  
Signature of committee chairman