A STUDY OF MECHANISMS BY WHICH HYPOXIA INFLUENCES NERVOUS ACTIVITY

by

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A THESIS

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My wife, "widowed" by my calling to science, is acknowledged for her stoicism and patience for the duration. Finally, I dedicate this thesis to Skip and Scott, my two sons, whose future is inexorably bound to mine.

TABLE OF CONTENTS

INTRODUCTION, PART I - THE PHYSIOLOGY OF NEURON CHAINS

INTRODUCTION, PART II - THE ENERGETICS OF ION METABOLISM

INTRODUCTION, PART III - EXPERIMENTAL DESIGN

OUTLINE RESULTS SECTION

OUTLINE DISCUSSION SECTION

LIST OF ILLUSTRATIONS

LIST OF TABLES

ORIENTATION TO THE PROBLEM

This research was begun as an attempt to estimate the energy cost of nervous activity. When the spinal cord of a frog is deprived of oxygen, reflex activity fails. This failure is presumably due to a deficit in the production of energetic intermediates necessary to maintain normal nervous function. It should be emphasized that, as is often the case, a relatively simple question has been found to consist of a large set of dependent subquestions. For instance, what is the functional stability of the isolated frog spinal cord over long time periods? Does the reflex failure during hypoxia occur in a reproducible and predictable pattern? Are the kinetics of metabolism related to function in such a way as to permit the pattern of failure to be modified by manipulation of experimental variables such as temperature and activity level? Neurons are elongated, irregularly shaped cells whose various parts may be some distance from other portions of the same cell. Can one expect a uniform response to hypoxia in all parts of the cell? Where in the reflex path does failure occur under hypoxia? What portions of the neuron are the most sensitive to hypoxia? Can hypoxic reflex failure be mimicked by conditions designed to manipulate specific metabolic pathways?

All of these questions are considered in the research reported herein. The problem is open ended in that most of these questions remain to be completely answered and continue to constitute the basis for additional research. However, this thesis provides, for the first time, the description and characterization of some metabolic correlates to nervous activity in a little-used preparation eminently suitable for studies of this kind. The experiments have provided a starting point for continuing the study of the relationship between metabolism and function in the central nervous system.

The background for understanding the mechanism involved in spinal cord hypoxic dysfunction lies in the literature of several fields. The extensive introduction to this study discusses pertinent details of the following subjects:

1. Neurophysiology of the spinal cord
2. Electrophysiology of spinal activity
3. Specific aspects of spinal cord hypoxia
4. Energetics of ion metabolism

The neurochemical aspects of ion metabolism are pertinent for the following reason: The initial hypothesis is that hypoxia primarily disturbs active ion transport and that the resultant neurophysiological changes can be explained on this basis.

INTRODUCTION

PART I - THE PHYSIOLOGY OF NEURON CHAINS

CENTRAL MECHANISM OF REFLEX ACTIVITY

What is a Reflex?

As described by Sherrington (1), a reflex is a "mindless act" wherein an organism, in response to a stimulus, performs motor activity in a stereotypic and reproducible manner. Even when the brain, the center of consciousness, is destroyed the dog responds to the "electric flea" by rhythmic scratching of the stimulated area, accurately localizing the irritation and performing the activity in the same fashion as if he were awake and healthy. A large number different unconscious, purposeful motor responses to various stimuli have been recorded. In every case the reflex results from a motor neuronal discharge in response to some stimulus.

Many of the reflexes that can be elicited from an animal appear to subserve specific purposes. Many are protective such as the corneal reflex, the ear twitch, the scratch reflex, and the flexion reflex. Many are adaptive, such as the various cardiovascular reflexes for maintenance of blood flow, pressure, and temperature control. Many reflexes are present as components of complicated adjustment of motor behavior. The stretch reflex which the physician commonly tests by tapping the patellar tendon is a component of postural regulation. Though integration of sensory activity to produce appropriate motor responses may occur on a subconscious basis the responses are obviously in accord with one"s conscious behavior, and may even be the basis for the execution of willful, conscious motor acts.

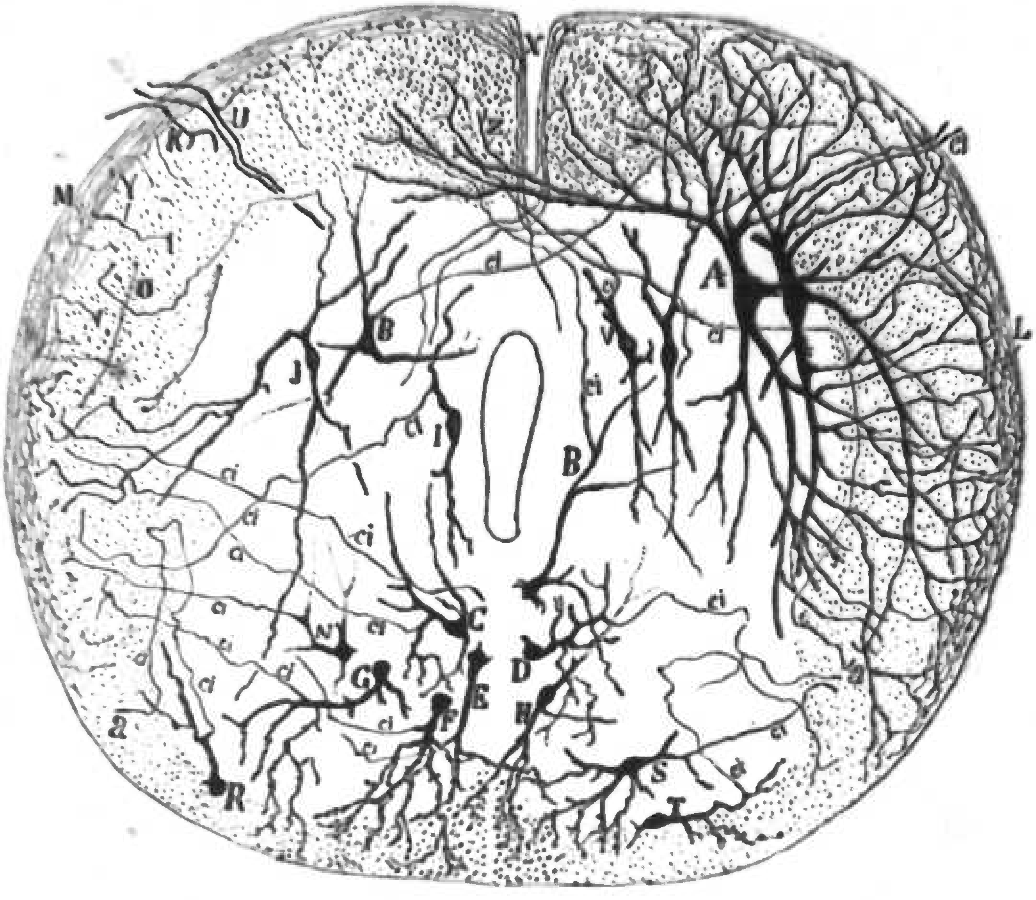
FIGURE 1. Spinal neurons of the toad (Bufo vulgarus).

A. Motor neuron - Note the extensive dendritic aborization. Dendritic branches form an extensive subpial plexus not seen in phylogenetically higher vertebrates.

B-T. Interneurons

The stippling is in the region of the white matter.

[From Sala y Pons, C. (2)]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CENTRAL MECHANISM OF REFLEX ACTIVITY

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Reflexes, especially the simple ones, provide a system to study the behavior of interacting neurons. Presumably all neurons, whether or not they are involved in reflex activity, interact in the same manner. There must be stimulus and the activated sensory neuron conducts to the CNS which integrates this information by specific anatomical pathways with other sensory inputs, and selected motor neurons are controlled in an appropriate manner by excitation or inhibition to generate a response.

THE ANATOMY OF SPINAL REFLEX PATHWAYS

From Figure 1 one may discern many prominent features of the internal structure of the frog spinal cord which are typical of all vertebrates (2,3). The motor neuron cell body lies in the ventral grey matter of the cord; its axon exits the cord bundled with other motor neuron axons to form the ventral root of the spinal nerve. The sensory neuron cell body is contained in the dorsal root ganglion (not shown). The central processes are bundled together in the dorsal root and enter the spinal cord on the dorso-lateral surface. The peripheral processes extend distally to receptor sites in the various organs. Thus the spinal nerve carries both the afferent and efferent fibers of the spinal cord. Neurons which lie completely within the spinal cord interpose themselves between other neurons and are called interneurons or internuncials. Junctions between neurons (synapses) are anatomically distinct structures most readily observed in detail with the electron microscope. The "wiring diagram" of the reflex pathways is a necessary adjunct to the complete understanding of the reflex process. However, there are some general principles in addition to anatomy that are essential to the understanding of reflex physiology.

Reflexes, as with any nervous function, depend upon the generation of action potentials in nerve fibers. Action potentials are normally initiated at the junction of the receptor and the afferent fiber. Impulse conduction in the reflex arc normally ends at the junction of the efferent fiber and the effector cell. The events that occur between these two extremes are complicated and varied, but much is known about their mechanisms and the structures that support them. The coordinated pattern of reflexly induced activity is the result of control exerted in the reflex path which determines *what*, *how much*, and *when* neurons are *inhibited*, *facilitated*, or *excited*. Various points along the reflex path are the site of control and integration of reflex activity.

AFFERENT CONDUCTION AND DISTRIBUTION OF ACTIVITY

Afferent Structure

The primary afferent axons of all sensory modalities undergo collateral branching upon entrance into the spinal cord and when the spinal cord is transected, reflex activity below the level of the section can still be generated from all forms of sensory stimulation (4). It seems likely that most, if not all, reflex afferents send collateral branches to ascending systems that subserve conscious sensation or suprasegmental reflex systems. For instance, muscle spindle afferents are known to synapse directly upon spinal motor neurons and internuncials. However, spindle afferents also invoke activity in the brainstem, cerebellum, and cerebral cortex (5,31). Collateral branching of afferents is probably a general rule and is the anatomical basis for the divergence and spread of incoming signals throughout the whole CNS.

Branching of the axon is not limited to the production of collaterals but each collateral branches again at its terminus. The aborized axon terminals are seen by light microscope to terminate in enlarged swellings called synaptic boutons. A single axon may distribute a number of synaptic boutons over the surface of a single postsynaptic cell to establish not one, but several synaptic connections. These synaptic connections are found on the dendrites, cell body, and terminal axons of the CNS neurons (6,7,8).

Electron microscopic evidence provides proof that there exists a cytoplasmic discontinuity between the pre-and postsynaptic cell. This conclusively establishes Cajal's "neuron doctrine" which states that all neurons of the CNS are distinct cells and that the CNS is not a syncytium of interconnected neural elements (9). On the basis of electron micrography synapses may be divided into two types. Type I synapses (axodendritic) are characterized by a 300 A cleft containing electron dense material and a thick postsynaptic membrane. Type II synapses (axosomatic) have a less densely staining cleft of 200 A width and an irregular thickening of the postsynaptic membrane. In both types, the presynaptic terminals contain prominent vesicles and mitochondria. The vesicles are seen to disappear with activity and are thought to contain the chemical transmitter agent secreted during activity. Some believe that the morphology of the vesicles indicates functionally whether they will secrete inhibitory or excitatory transmitter (10,11).

Conduction in Afferents

Before progressing further into synaptic transmission it is important to consider the factors involved in the electrical manifestations of nervous activity. There are three reasons for delving into details of the physics involved in electrical potentials of nerves. First, synaptic transmission cannot occur unless an action potential is propagated into the synaptic bouton. Anything that can modify the action potential in the afferent terminal will influence synaptic transmission and hence, reflex activity. Secondly, certain structure-function relationships can be predicted from knowledge of the physical parameters for conduction. Lastly, given understanding of certain physical principles it is possible to hypothesize specific changes in the efficacy of conduction in reflex paths during abnormal conditions such as hypoxia or ouabain poisoning.

There are two important aspects in the spread of electrical disturbances in nervous tissue. One of these involves the passive physical properties of the structure, the other involves active physicochemical changes in and around the membrane. In its passive behavior, the neuron and its processes can be treated as a relatively poorly conducting wire or insulated cable. In its active behavior, the genesis of the action potential depends upon operational molecular systems.

Among the important parameters of cable properties are the various resistances to passage or conduction of current. There is resistance along the outside surface of the axon which is a function of the conductivity of the bathing solution. The membrane itself presents resistance to current flow dependent upon its molecular structure. Finally, the axoplasm has a characteristic resistivity. Since most vertebrate nerve fibers have a small cross-sectional area, the internal resistance is quite high.

resistance = R L/A

R=resistivity of axoplasm
L=length
A=area of cross section

The external resistance is quite low relative to the internal resistance because the volume available for conduction is normally large relative to the volume of the nerve fiber. Axoplasmic volume resistivity (Ri) has been estimated from the ionic composition as 200 ohm cm (12). The volume resistivity (Ro) of physiological saline is 50 ohm cm. The membrane resistance (Rm) is of the order of 1000 ohm cm^2 (13).

A local change in potential across the membrane will result in current flow through the membrane and along the outer and inner surfaces of the membrane. If the axon is modeled as a purely resistive network an equation may be derived to express the transmembrane potential along the axis of the fiber in either direction from the locally induced potential change.

$$Vm = Vmmax\ (1-e^{\frac{-x}{\gamma}})$$

Where Vm=transmembrane potential
x=distance from point of depolarization
$\gamma = \frac{Rm}{Ro+Ri}$

The symbol γ is sometimes called the "length constant" and is the distance over which the transmembrane potential changes by a factor "e" (base of natural logarithm). An unmyelinated axon with the physical constants indicated above and with a diameter of 1 μ will have a length constant of 0.11 mm. This means that the transmembrane potential will be 63% of the resting potential 0.11 mm from the point of depolarization. When dynamic measurements are made of such electronically conducted potentials one can observe a time lag between the peak of depolarization and the peak voltage at a point distant from it. In order to account for this temporal behavior a membrane capacitance must be included in parallel across the membrane resistance. Consequently, as electrical disturbances are conducted along the axon, the amplitude and time course of the potential change at a point distant from the source will be a function of the resistance and capacitance characteristics of the axon. Since membrane resistance and capacitance is relatively constant from fiber to fiber, the axoplasmic resistance is the important variable. (However, the action potential generating mechanism can accommodate to depolarization if the rate of change of potential is too slow. In this case, excessive membrane capacitance would prevent the generation of an action potential.) Axoplasmic resistance varies inversely with the fiber diameter. Small fibers produce smaller and slower electrotonic potentials than large fibers.

The passive electrical properties of axons are distinct from the potential changes that are the result of those molecular events localized in the membrane which underly the genesis and propagation of the action potential. The action potential is generated by an as yet unknown mechanism which controls transmembrane ionic fluxes (14). The action potential is initiated by current flowing outward through the membrane and is an all-or-none event that lasts about one millisecond. Once initiated, the action potential is electrotonically conducted along the membrane away from its source. The resultant current flow in adjacent unexcited membrane of the same axon begins to depolarize this membrane and, when depolarization reaches threshold the adjacent membrane becomes involved in the all-or-none response. Obviously the speed with which an action potential propagates from point to point along the membrane is dependent upon the rate of rise of the electrotonically conducted potential advancing before the front of the action potential. The primary determinant for attenuation of the action current is the axoplasmic resistance. Thus the smaller the diameter of the axon the greater will be the resistance to current flow and the slower the potential rise to threshold. The slower this rise, the slower will be the conduction velocity of the action potential. Theoretical calculations from data of passive electrotonic properties accurately predict the conduction velocities of various sizes of squid axon (13,14). As expected the propagation velocity is decreased when the axon is bathed in oil to increase the external resistance. Similarly, the propagation velocities are increased when a wire is inserted into the axoplasm to decrease the axoplasmic resistance to electrotonic conduction.

Myelinated axons are covered by a myelin sheath which is interrupted at intervals of 200-1000 μ. This myelin sheath greatly increases the resistance between the axoplasm and interstitial fluid (10^5 ohm cm^2) (13). At the nodes of Ranvier, the region between the interrupted cylinders of myelin, the resistance between the axoplasm and interstitial fluid is least because of the absence of the myelin sheath. Current flow through the internodal region is not sufficient to generate action potentials in the internodal membrane. However, current flow from node to node normally exceeds, by a factor of 5, that amount required to bring the nodal membrane to threshold levels of depolarization. The ratio of action current to current necessary to elicit an action potential is defined as the <u>safety factor</u>. Conduction velocities in myelinated nerve are higher than in non-myelinated nerve because the action potential jumps from node to node instead of being delayed by propagation along the whole length of the internodal membrane. If the safety factor for conduction is five, the minimum current for excitation must be at least 20% of the action current. The length constant for a 1 μ myelinated fiber is an order of magnitude greater than for an unmyelinated fiber of similar diameter (1.1 mm). The maximum distance from one node to the next which would result in an action current of 20% would be about two length constants for a 1 μ myelinated fiber or about 2 mm. In addition to increasing the length constant, the myelin sheath considerably reduces the capacitance of the axon and thereby increases the rate of rise of electrotonic potentials (dv/dt) at the adjacent node. Thus, a myelinated fiber of a given diameter has a greater propagation velocity than an unmyelinated fiber of the same internal diameter.

One could predict that smaller diameter myelinated fibers would require shorter internodal distances in order to maintain a reasonable safety factor for action potential propagation (17). Such a correlation

($r=0.62$) between internodal length and diameter has already been shown for bullfrog sciatic nerve (18). The following equation expresses this relationship.

$$L=0.146 \times 10^3 d$$

where:
L=internodal length
d=fiber diameter

Measurements of nerve electrical potentials may include electrotonic as well as action potentials. Furthermore, small diameter fibers contribute less to the recorded potential than larger diameter fibers (15). In some experiments described in this thesis, electronically conducted potentials of greater magnitude than action potentials were recorded. It is important, in the analysis of electrical recordings, to recognize the difference between potentials electrotonically conducted to the recording electrode and those that are the result of action potentials at the electrode.

From a teleological point of view, it is reasonable that the myelin disappears in the fine branches of the terminal axon. Were the branchlets myelinated, extremely short internodal distances would be necessary. Since the terminal branchlets are not myelinated, action potential invasion of the terminus is more secure. Little is known about the cable properties of these fine elements, but it is reasonable to expect that propagation velocity through the terminal branchlets is considerably less than in the parent axon. No information exists concerning the stability of cable properties under adverse conditions such as hypoxia. However, the vulnerability of normal ion distribution and metabolism to hypoxia will be discussed below in detail. The above sections lay the ground work for the succeeding discussion of control and integration of reflex activity.

SYNAPTIC TRANSMISSION AND INTEGRATION OF REFLEX ACTIVITY

In vertebrate nervous systems, any particular neuron functions by influencing the excitability of postsynaptic neurons. A postsynaptic neuron such as the motor neuron receives synaptic connections from a large number of different afferents and internuncials. The synaptic boutons appear to be structured uniformly and vary slightly with motor neuron size (4). These synaptic boutons cover 40% of the surface of the soma and dendrites and may number as many as 5500 on a single motor neuron in the mammal (4). Direct cell counts in the L7 segment of the dog spinal cord indicate that there are 375,000 cells; but there are only 1200 fibers in the L7 dorsal root and 6,000 in the L7 ventral root (4). The large number of internuncial and tract cells between the input and output could indicate capability for a considerable amount of integration and processing of neuronal information.

In the vertebrate nervous system the excitability of the vast majority of postsynaptic cells is changed by a chemical substance secreted from the presynaptic terminals. (The arguments for chemical rather than electrical transmissions at these synapses are universally accepted and not pertinent to this paper.) At these chemically operated synapses the action potential in the presynaptic terminal branchlet causes release of the transmitter agent into the synaptic cleft. Little is known about the mechanisms by which electrical activity causes secretion. The ion Ca^{++} which has been shown to couple membrane excitation to the contractile system is also necessary for excitation-secretion coupling in the presynaptic terminal (10,16). After the transmitter is released, it diffuses across the synaptic cleft where it combines with a receptor site to generate the postsynaptic response. At the same time, an enzyme in the synaptic cleft breaks down the transmitter substance and the products are re-assimilated by the presynaptic structure to be resynthesized into transmitter agent. The problems of transmitter identification in the vertebrate nervous system are exceedingly difficult to resolve, and as a result, only fragmentary ambiguous and indirect evidence in favor of any particular substance has been made available.

Acetyl choline and related enzymes are distributed throughout the CNS; but as yet it is best identified as the transmitter exciting the Renshaw cell of the spinal cord (10,17). Epinephrine and catecholamine enzyme systems are also widely distributed but no CNS transmitter function has been proven. Other likely candidates for transmitters include serotonin, histamine, gamma amino butyric acid (GABA) and glycine (10,17).

Vertebrate transmitter substances acting at specific receptor sites can be excitatory or inhibitory to the subsynaptic cell. Furthermore, a particular neuron probably secretes only one kind of transmitter from all of its terminals (8). It is difficult to imagine the enzyme systems for one kind of transmitter localized to one collateral branch, and a different system localized to another branch. This is especially true if the enzymes are synthesized in the neuron nucleus and distributed to the terminals by axoplasmic flow. A good example is the spinal motor neuron. This neuron secretes acetyl choline at the skeletal myoneural junction and probably does so at the recurrent collateral to the Renshaw cell. Another example can be drawn from the known physiology of primary afferents involved in monosynaptic reflexes. If a primary afferent causes monosynaptic motor neuronal discharge, it must be excitatory to that motor neuron. However, collateral branches of the primary afferent inhibit discharge in heteronymous motor neurons. If the collateral branches secrete only excitatory transmitter, then there are two possibilities to account for the inhibition. First, there could be an internuncial cell interposed between the afferent collateral and the heteronymous motor neuron. This internuncial would be excited by the afferent and in turn secrete an inhibitory transmitter. Inhibition of heteronymous motor neurons occurs with latency suggesting a disynaptic pattern. There is also histologic evidence to support the first explanation of how activity in a single afferent can cause excitation in one motor neuron pool and inhibition in another (18).

The second possibility is suggested because of recent histological evidence that an afferent can make monosynaptic connections to heteronymous motor neurons (19). The receptor site mechanism may determine whether or not a given transmitter is excitatory or inhibitory. This concept is supported by the observation that certain molluscs have giant ganglion cells whose response to directly applied micro doses of acetyl choline varies from depolarization to hyperpolarization as the micropipette is moved across the surface of the cell membrane (20).

Micro-electrodes inserted into the postsynaptic cell can be used to detect the response to synaptic activity. Synaptic excitation of neuron results in a characteristic potential change in the subsynaptic membrane potential which is conducted electrotonically throughout the neuron and may be detected by the micro-electrode in the cell body. The excitatory postsynaptic potential (EPSP) is graded in nature and unlike the all-or-none characteristic of the action potential. Stimulation of a few excitatory afferents may produce a small EPSP after a delay of about 0.5 msec. This delay includes the time for the transmitter secretion and diffusion from the presynaptic terminal to the postsynaptic membrane. It also includes the time from combination of transmitter-receptor and the reactions that generate the beginnings of the EPSP. The EPSP is apparently the result of a nonspecific increase in permeability of the subsynaptic membrane, but the molecular processes involved are unknown (17). As the number of activated presynaptic terminals is steadily increased larger and larger EPSP's are produced until the current flow from the EPSP's is sufficient to depolarize the postsynaptic cell to threshold.

Careful examination of the micro-electrode recordings from the soma of impaled motor neurons reveals that there are three phases of potential change from a synaptically produced discharge (21). The first phase is the electrotonic depolarization caused by the EPSP. The second phase is the electrotonic conduction through the cell body of the action potential generated at the initial segment of the axon. The last phase of the recorded discharge is the actual propagation of the action potential into the membrane of the soma and portions of the dendritic tree. Even though the synapse may be on the cell body or basal dendrites of the postsynaptic cell, the action potential is initiated in the initial segment of the axon.

Inhibitory postsynaptic potentials (IPSP) are observed as a hyperpolarization of the postsynaptic cell. IPSP's may be the result of a specific increase in permeability to K+ or Cl- ions but not to Na+. Such potentials serve to increase the difference between the membrane potential and the level of depolarization necessary to trigger an action potential (threshold potential). The hyperpolarization of an IPSP sums algebraically with the depolarization of a concurrent EPSP and the influence on the excitability of the initial segment is the resultant of these two opposing actions. Since the level of threshold for impulse generation is a relatively constant feature of a given CNS motor neuron, the excitability of a neuron from moment to moment is a function of the relative magnitude of concurrent EPSP's and IPSP's. Duration of a postsynaptic potential depends upon the amplitude and time constant for electrotonic conduction as well as the duration of transmitter action. For example, a 3 mv motor neuronal IPSP may reach its peak in two msec and decay exponentially over the next 20 msec. The time course of EPSP generation is similar and is almost the mirror image of the IPSP in any one spinal motor neuron (17).

Considerable evidence has been amassed in the last few years concerning presynaptic control over synaptic transmission. Fluctuation of discharge in motor neurons has been observed in monosynaptic reflexes even though no IPSP or change in threshold could be detected at the motor neuron. Instead the threshold for discharge of primary afferent terminals changes (22). Following dorsal root stimulation electrotonic potentials recorded from dorsal roots indicate that the afferent terminals remain depolarized for some time relative to the axons in the dorsal roots. Stimulation in the region of the afferent terminals supports this observation because the terminals appear to be more excitable, that is, nearer threshold (23). It is apparent from such studies that depolarizing synapses on primary afferent terminals may induce primary afferent depolarization which is accompanied by reduction of the amount of excitatory transmitter released from those terminals (16). Two mechanisms have been suggested by which depolarization of afferent terminals could produce a reduction in the amount of transmitter released. The first suggests that depolarization of one or more of the fine terminals branchlets could produce blockade of the afferent active potential before invasion of the synaptic bouton (24). The blockade of a number of synapses from invasion by action potential would reduce the amount of transmitter released which in turn would reduce the magnitude of the EPSP. The second suggests that the amount of transmitter released from the presynaptic membrane is a function of the amplitude of the action potential (25). By this mechanism, all of the terminals could be invaded by an action potential but presynaptic depolarization would reduce the amplitude of the action potential and hence the amount of transmitter released into the synaptic cleft. This latter hypothesis has support from direct measurements of transmitter release in the giant squid synapse (16). Since the end result of the process is a reduction in output from less vigorously excited postsynaptic neurons it has the appearance of inhibition and has been misnamed presynaptic inhibition.

It is thus apparent that a reflexly induced response involves a variety of processes which occur at various portions of elongated cells. None of these processes is completely understood and all but the passive physical characteristics may be susceptible to influence through experimental manipulations. Before proceeding to an analysis of these possibilities, it is necessary first to consider how the recorded potential signs of nervous activity may be interpreted in a rational and reliable fashion.

PRINCIPLES OF INTERPRETATION OF ELECTROPHYSIOLOGIC RECORDS

With appropriate instrumentation, it is possible to detect afferent sensory activity and efferent motor activity simultaneously. by the proper placement of recording electrodes. Furthermore, simple reflex activity can be artificially induced by electrical stimulation of afferent nerves and the results detected by recording the electrical responses in the spinal cord or in the motor nerve. It is possible, therefore, to elicit and detect reflex activity without the presence of receptors and muscles.

The literature abounds with descriptions of techniques for the manufacture of gross and micro stimulating and recording electrodes (28,29,30). The literature also contains descriptions of a vast number of different nervous system preparations at all phylogenetic levels (27). It is not reasonable nor particularly pertinent to discuss them here. What is pertinent is to present some of the typical recordings obtained by electrophysiological examination of the spinal cord reflexes. Associated with this presentation will be an exposition of the basis upon which these signals are analyzed. This section will serve as an adjunct to the methods involved in the experimental portion of this thesis.

Figure (2A) is an example of the electrical potential changes that are observed in the ventral root as a result of electrical stimulation of the homolateral dorsal root in the same segment. In effect, a reflex discharge of motor neurons is produced from which

FIGURE 2. Spinal Reflex Discharges in the Cat

Part A (a-d): Successive reflex discharges in the ventral roots in response to stimulation of homolateral dorsal root. The arrows indicate onset of monosynaptic and polysynaptic reflex induced discharge of motor neurons respectively.
[From Renshaw (32)] .

Part B: Ventral root discharge following stimulation of the spinal grey matter and dorsal root.
a. Response to spinal grey stimulation. The m wave is due to discharge as a result of direct excitation of the motor neuron. The s wave is due to synaptic excitation of the motor neurons.
b. Response to submaximal dorsal root stimulation.
c. Response to both spinal grey and dorsal root stimulation.
Note the facilitation of the s wave.
[From Renshaw (32)].

Part C: Reflex discharges recorded from the ventral root in response to peripheral nerve stimulation.
a. Response to stimulation of nerves to the gastrocnemius.
b. Response to stimulation of the sural nerve.
Similar conduction distances and amplification were used in obtaining both records. Note the differences in latency and synchrony between the monosynaptic (A) and polysynaptic (B) responses.
[From Lloyd (33)].

Part D: Reflex discharges in the ventral root in response to dorsal root stimulation. This record should be compared to Part C. It illustrates the difference between patterns of discharge from stimulation of peripheral (C) and dorsal root (D) fibers. Both monosynaptic and polysynaptic responses are distinguishable. Time marks equal 5 msec.
[Record from Lloyd (33). Reproduced from Brazier, M. (34)].

Note: Negativity is upward in all traces.

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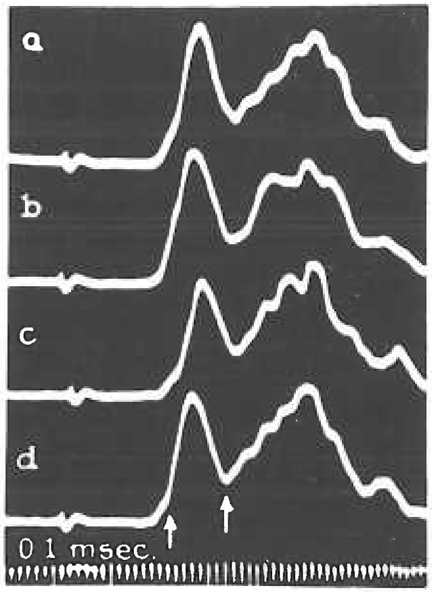

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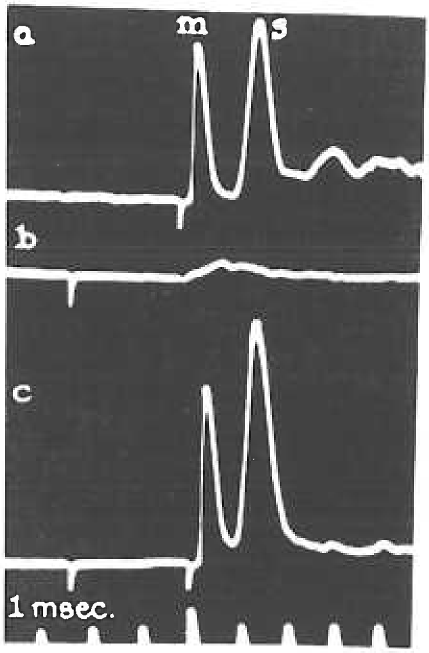

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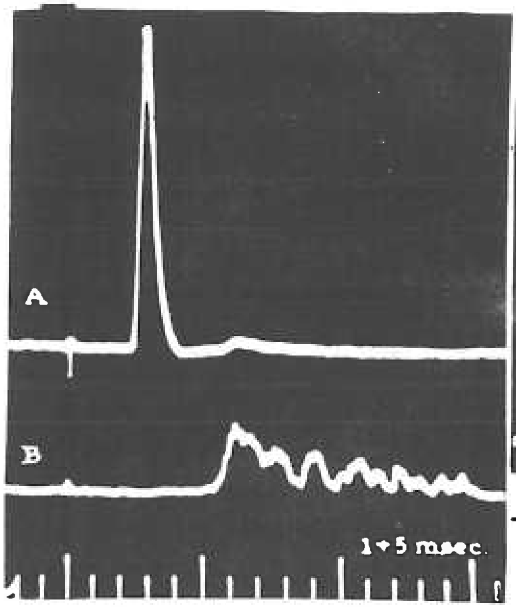

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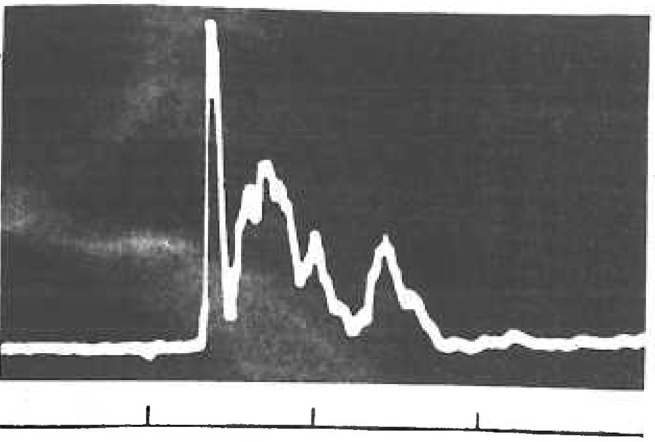


much information may be derived. For instance, one can measure about one millisecond from the time of stimulus to the onset of ventral root discharge (latency). If correction is made for conduction time in the roots, the delay at the synapse is estimated at 0.6 msec. (32). When the ventral grey matter is directly stimulated a record similar to Figure (2B) may be obtained. The response labeled m is from motor neurons discharged directly by the stimulus and s is from motor neurons discharged synaptically by directly stimulated presynaptic fibers. The minimum interval between responses is also about 0.6 msec. Renshaw (32) concluded from these data that the initial synchronous volley in the reflex discharge (Figure 2A) is due to monosynaptically activated motor neurons. The subsequent fluctuation of potential is due to polysynaptic, asynchronous activation of motor neurons by internuncials. Lloyd (33) showed that the patterns of reflex discharge depended upon the size and source of the stimulated afferent fibers (Figure 2C, 2D). These classical experiments are examples of how electrical responses can be used to make inferences about central synaptic relationships.

With the development and improvement of micro-electrode techniques it became possible to record potentials from small regions within the spinal grey matter. These focal potentials were subjected to analysis by Brooks and Eccles (35).

With a micro-electrode in the ventral spinal grey matter it is possible (as illustrated in Figure 3) to differentiate the response

FIGURE 3

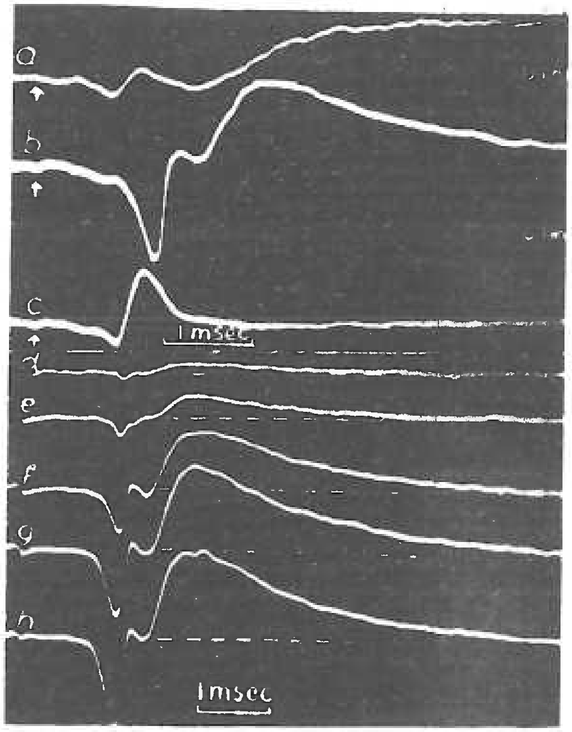
Part A - From Figure 2 by Brooks and Eccles (35)

Action potentials set up by a single orthodromic volley initiated in the quadriceps nerve and entering the spinal cord through L_6DR. Potentials a,b and c are set up in the same experiment by stimuli just maximal for the large afferent fibres. Arrows indicate stimulus artifacts. a. Potential recorded from the L_6VR which is insulated by suspension in the paraffin, the electrodes being at the point of emergence from the spinal cord and at the cut end. (cf. 8). b. Potential recorded between an indifferent electrode and a needle electrode in the quadriceps nucleus. In this and all subsequent records of focal potentials, negativity of the focal electrode is shown as an upward deflection. c. Potential recorded from the L_6DR at the levels of the needle electrode. d-h. Potential recorded as in b but in another experiment with deep anaesthesia. (90 mg. nembutal per kg. given intravenously during the preceding 3 hours). In d stimulus is just above threshold and its strength was increased by about 10% for each subsequent record, being in h maximum for the large afferent fibres. Note slower time scale than for a-c. Same potential scale b-h.

Part B - From Figure 1 by Brooks and Eccles (35)

Schema of lines of current generated by the propagation of an impulse into the terminal end of a fibre immersed in a volume conductor (cf. 23, Fig. 7). The active phase of the impulse is hatched in and its crest is indicated by an arrow. It shows that the current flow at the terminal continues to be outward until the arrival of the crest. The broken lines show iso-potential contours. b. As in a, but to show that after arrival of the crest at the terminal the flow continues to be inward even after the terminal has recovered from the active phase (the innermost current line). c. Schema as with a, but showing lines of current flow generated when the soma of a motoneurone (the circle) is depolarized relative to its axon, which is shown emerging from a ventral root suspended in an insulating medium. The potential field is also drawn (broken lines), the lined marked 0 being that of the indifferent electrode.

A



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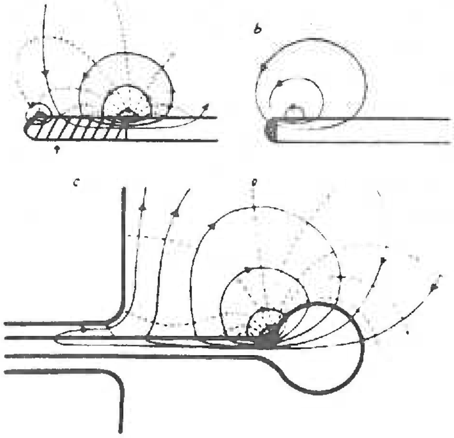


of afferent (presynaptic) elements from the response of motor neurons (postsynaptic).

The basis for detailed analysis of the shape of the wave forms derived from focal micro-electrode experiments has been clearly described by Renshaw et al. (36) and augmented by Brooks and Eccles (35). The five basic premises are as follows:

> 1. The potential at a point in a conducting medium due to the existence of activity in portions of a cell which is at a distant part of the medium is small (either approaches or actually is zero as the distance of the point from the cell increases). Therefore an electrode remote from a nerve center may be considered as indifferent with regard to electrical activity in that center.
>
> 2. There is no external electrical field set up and therefore no current flows in the medium about a cell so long as every part of the surface of the cell is at the same potential. Even if the potential difference between the outside and the inside of the cell should change greatly, no external field would be set up if the change took place uniformly and simultaneously over the entire surface.
>
> 3. An external electric field is set up when a potential difference occurs between any two regions of the surface of the cell. Current flows from the regions of high potential to those of low through the surrounding medium and returns within the cell. Points in the medium close to a region of the cell surface which is at a relatively low potential are negative to the distant point p. Points close to a region which is at a relatively high potential are positive to the point p.
>
> 4. The difference in potential between any point n near the cell and a distant point ′(which is at approximately average or zero potential due to the condition described in (c) decreases very rapidly as the distance of n from the cell increases. The density of current becomes rapidly greater as an active portion of a cell is approached because of the decreasing volume through which the action current may flow. Consequently, assuming approximately constant specific resistance of the medium, the IR drop becomes rapidly steeper as the active region (or an adjacent inactive region) is approached.
> (Renshaw, B., et al. (36).

5. When current is flowing through the membrane into the conducting medium, a focal electrode on the surface will be positive to the indifferent electrode in direct proportionality to the density of the penetrating current. The converse is true for the negativity of the focal electrode when the current is flowing inwards.

Figure 3B is a diagram from Eccles that illustrates these points. The features of traces in Figure 3A can be interpreted as follows. The initial positive wave of the trace signals the distant depolarization of the afferent fiber. The potential rapidly swings negative as the wave of afferent activity spreads into the region of the micro-electrode. A slight positive dip is often seen as the afferent activity passes beyond the electrode, but this is immediately followed by a large persistent negative potential produced by postsynaptic depolarization in the region of the micro-electrode. Therefore, with one electrode the afferent and efferent activity of the reflex can be monitored.

Obviously, a multitude of stimulus recording combinations can be arranged depending upon the investigator's experimental design. Since the spinal cord contains distinct regions, sensory, motor, ascending, descending, internuncial, etc. signals from different regions will predominate depending upon the placement of the electrodes. It is even possible to record from within single nerve cells (37).

Potentials led from the dorsum of the cord were first recorded by Gasser (38). Figure 4 clearly shows these early and obviously primitive records. The rapid triphasic spike is a signal of the afferent volley from dorsal root stimulation. The subsequent negative wave is produced principally by internuncial discharge, but

FIGURE 4

Part A - From Fig. 9, Gasser (38)

a. Response to dorsal root stimulation recorded from the dorsum of the cat spinal cord. Simultaneous observation of the ipsilateral hind limb revealed no reflex movement.

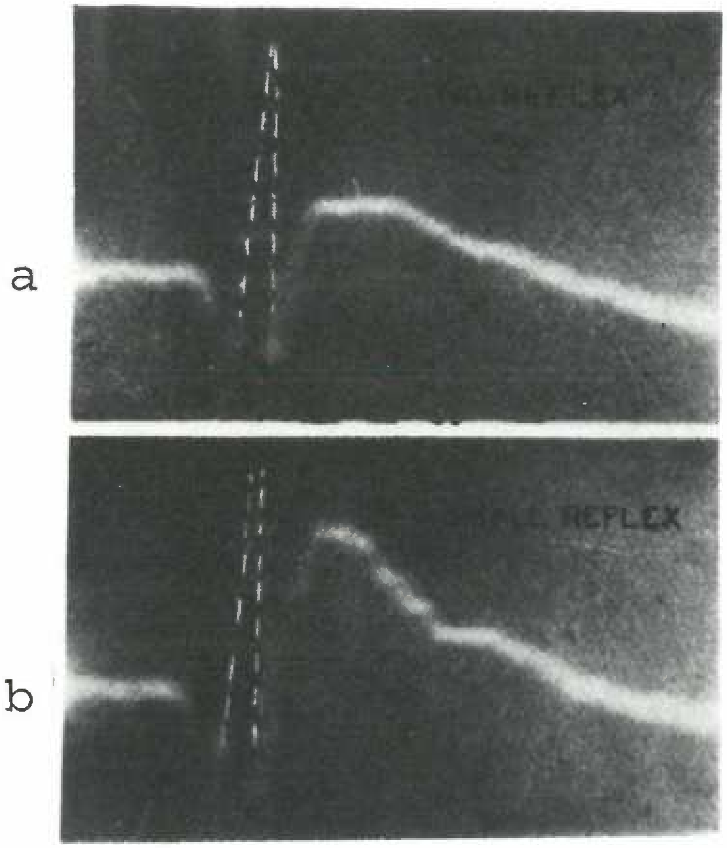
b. As in a above but with stronger stimulation and with resultant hind limb flexion. Arrow indicates the onset in time of muscular activity.

Part B - From Fig. 5, Gasser (38)

a. Normal cord dorsum response to dorsal root stimulation.

b. Cord dorsum response to dorsal root stimulation after blockage of synaptic activity by hypoxia.

A



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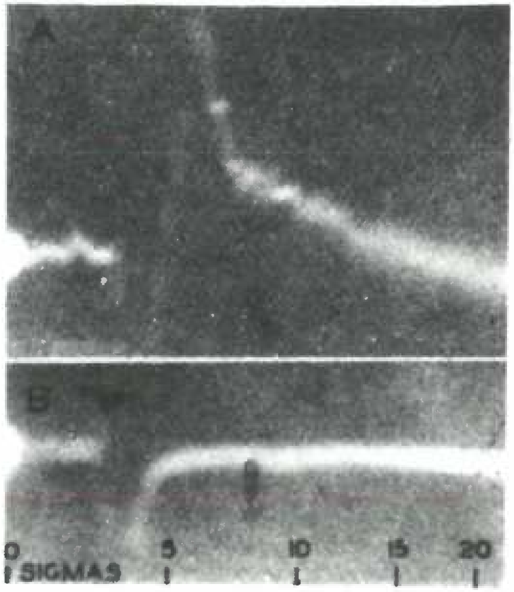


may be augmented by motor neuronal discharge as in trace 8. The effect of hypoxia is shown in the second part of the Figure where the negative or postsynaptic wave disappears even though the afferent signal persists. (Note that the amplitude of the deflection and writing speed of the oscilloscope phosphor does not permit direct observation of the afferent spike. Its presence is inferred from the positive deflections and break in the baseline). These are important observations because they demonstrate a difference in the susceptibility of peripheral and central nervous systems to hypoxia.

EFFECTS OF HYPOXIA ON SPINAL REFLEX ACTIVITY

Techniques and Gross Observations

The rapidity with which hypoxia causes loss of consciousness has been the subject of many and varied studies. The importance of this kind of study is obvious. Firstly, most deaths, regardless of etiology, terminate by asphyxia of the central nervous system. Secondly, the CNS is remarkably sensitive to dysfunction produced by hypoxia, probably more so than any other tissue (39). [However, see Lindenberg (40)]. It is the purpose of this thesis to investigate the mechanisms by which hypoxia causes dysfunctions in the CNS. The portion of the CNS to be examined in detail is the spinal cord. Consequently, this section will consist of a critical review of papers dealing primarily with hypoxia and spinal reflexes.

A number of papers offer an excellent review of the literature on the subject of hypoxia and spinal reflexes (41,42,43). Krogh (42) cites the earliest publication as that of Nicoli Stenosis (1669), who observed convulsions as the consequence of aortic ligation. It is interesting to note that this technique is used today as a convenient means of inducing hypoxia in the spinal cord. Other techniques include ischemia by pressurization of the spinal canal above the blood pressure, cessation of artificial ventilation, and ventilation with gas mixtures low in oxygen (42). The latter technique does not result in the accumulation of CO_2 in the spinal cord tissue. However, it does result in a general hypoxia whereas ligation of the abdominal aorta will not produce cerebral ischemia and resultant loss of spontaneous ventilation. Instead abdominal aortic ligation causes ischemia in the lumbar sacral region of the spinal cord. Consequently, when chronic experiments are performed, aortic ligation is preferred. A possible disadvantage is that aortic ligation does not produce absolutely complete ischemia (42) and may cause pH changes by the accumulation of CO_2. Even though CO_2 has been shown to depress monosynaptic reflexes, Kirstein (44) observed that patterns of reflex failure in cats are similar regardless of how the anoxia is produced. Therefore, the effect of ischemia on spinal reflexes is considered to be primarily the result of stagnant hypoxia (44).

Histologic damage and clinical manifestations of dysfunction are dependent upon the degree and duration of hypoxia. Furthermore, change in histology and dysfunction may continue up to two weeks following the hypoxic period.

Histologic examination of the spinal cord after signs had stabilized, two to three weeks following an ischemic episode, revealed amounts of damage dependent upon the duration of the hypoxia (42,45,51). Van Harreveld (45) correlated these results with clinical observation of the experimental animals. Pressure induced ischemia of cat's spinal cord for 15-20 minutes did not result in marked alteration in reflex activity or gross histological damage up to two weeks after the episode. If the ischemia was prolonged to between 25-35 minutes, spasticity (considerable extensor tone, active tendon reflexes, and clonus) was observed. Spinal cords from these animals showed gross histological changes. There was nearly complete absence of the cells surrounding the central canal, and considerable damage in the central and medial regions of the ventral grey matter. In the peroneus-tibialis motor pool 93% of the nerve cells were destroyed and the surviving few were the larger cells on the periphery of the nucleus. However, if one assumed that the larger cells were motor neurons, post asphyxial cell counts would indicate about 20% of the motor neurons survived. Fiber counts of L_7 ventral root indicate that approximately 30% of the motor neurons survive indicating that some of the small surviving neurons may be gamma efferent motor neurons (41). Ischemia for longer periods (up to one hour) produced a flaccid paralysis in the experimental animals and even greater histological damage.

According to Van Harreveld (53) there is a stereotypic pattern of hind limb tone changes in the two weeks following a pressure induced ischemia. Cats exposed to 25-35 minutes of ischemia pass through three stages of hind limb extensor tones. The "initial tone" develops 6-20 minutes after the end of the ischemia and lasts 15 minutes. The ensuing flaccidity is replaced by a "secondary tone" 6-12 hours later. This secondary tone lasts 1-2 days after which it may gradually disappear. Within 10-14 days the "late tone" develops and this rigidity persists for the duration of the animal's life. If the duration of the ischemia is longer than 35 minutes, the late tone does not develop.

Though this work seems fairly systematic, comparable studies by Krogh (42) do not show such well defined relationships of damage to duration of ischemia. For instance, flaccid paralysis was seen with 20 minutes of aortic clamping and 15-17 minutes of ischemia resulted in various degrees of cell damage and spastic paralysis. Both studies are in agreement in that motor neurons seem to be less sensitive than the internuncial population and that histologic damage is greatest in the more central portions of the grey matter. Gelfan (46) reports that motor neurons may lose 66% of their dendritic length after a 30 minute ischemic episode. Although he interprets this to be the result of denervation by internuncial death, one is unable to assess the possibility that the dendrites were destroyed as a direct result of hypoxic damage.

Acute hypoxia produces a rather stereotypic pattern of reflex failure. Augmentation of excitability in the initial stages of hypoxia has been described by several of the very early investigators in this field. Sherrington (47) wrote that "a certain degree of hypoxia favors the elicitation of the scratch reflex." Kaya and Starling (48) observed blood pressure increases and skeletal muscle spasms during the onset of hypoxia and concluded that spinal "motor centers" must be excited prior to their ultimate depression and failure. Tendon reflexes and flexion reflexes also show an initial augmentation (41). However, reflex activity in mammals evoked by muscle stretching or muscle nerve stimulation disappears within 35-45 seconds after the hypoxia begins (41). Flexion reflexes from superficial peroneous may survive 15-20 seconds longer. If the dorsal root is stimulated, reflex motor neuron discharge may survive 3-4 minutes. It has been shown that mammalian peripheral nerve survives hypoxia for 30 minutes or more at body temperatures (49,50). Consequently, the longer survival time of the dorsal root induced reflex compared to the motor nerve or tendon reflex is probably due to the activation of larger numbers of spinal neurons than can be excited by peripheral nerve stimulation. The inevitable failure of reflexes during hypoxia must also be due to inexcitability of spinal cord neurons. Recovery of excitability occurs after release from the ischemia. However, this recovery may only be temporary as in the case of preparations made ischemic for periods in excess of 35 minutes. In this case there develops a "secondary tone" after 6-12 hours, but the fact that this is followed by a persisting flaccidity indicates that some neurons eventually doomed to die are able to temporarily resume function. Van Harreveld believes that the initial episode of acute hypoxia results in depolarization of elements in the reflex pathway. Restoration of oxygen may result in rapid repolarization of these neurons but damage to "enzyme systems essential to life" results in eventual cell death (41,45). This cell death may take 48-96 hours before it is histologically visible (45).

These gross measurements of the effect of hypoxia on spinal reflexes are complicated and provide no indication of the mechanisms involved in reflex failure. An analysis of the electrophysiologic measurements of hypoxic reflex failure is presented in the next section. What is needed is an indication of the intraspinal events during hypoxia and an estimate of the most sensitive element in the reflex path to hypoxia. In this manner it will be possible to get a better idea of the mechanisms involved in hypoxic reflex failure.

Electrophysiologic Characteristics of Hypoxic Reflex Failure

Survival of reflex discharge in the ventral roots has been monitored by a number of investigators (44,54,55,56). As might be expected, the initial augmentation of the reflexly induced muscular activity, described earlier, is represented by an increase in the amplitude of potentials recorded from the ventral roots during the initial stages of hypoxia (44,56). Monosynaptic reflexes were observed to survive longer than polysynaptic reflexes during the acute phase and during recovery from prolonged hypoxia (41,44). However, because of differences in experimental design and inadequacies of data presented, it is difficult to get a good estimate of the survival times of reflexes with differing complexity.

Kirstein (44) reported that when peripheral nerves were stimulated to produce reflex motor neuron discharge there was an initial depression in the amplitude of the electrically recorded monosynaptic response at the onset of hypoxia. This was followed by augmentation and then subsequent failure of the reflex discharge. Augmentation of the polysynaptic reflex was not always seen. Figure 5 has been taken from Kirstein's paper to illustrate the time course of the potential fluctuations during ischemia and hypoxia. It may be noted that the survival times of these reflexes were longer with nitrogen hypoxia than with aortic occlusion. Assuming that barbiturates selectively depress internuncials, Kirstein concluded that the Dial sensitive initial depression, and late augmentation of the polysynaptic reflex was due to internuncial activity. The initial depression of the monosynaptic reflex activity was abolished by a 40 mg/kg dose of Dial, but in this case, the following augmentation was not. Consequently, Kirstein concluded that augmentation of monosynaptic excitability during hypoxia may originate from postsynaptic structures. Kirstein further argues that polysynaptic failure occurs in "presynaptic structures" since monosynaptic excitation of motor neurons was still possible after polysynaptic failure had occurred. Perhaps this investigator meant to say that internuncial transmission is more susceptible to hypoxia than the primary afferents or motor neurons. In any case, Kirstein does not

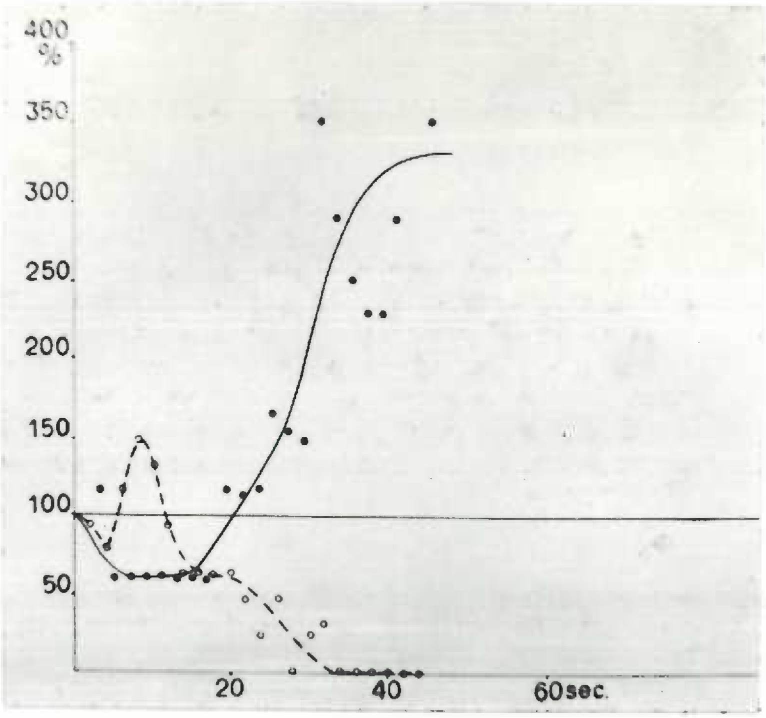
FIGURE 5. Effects of Hypoxia and Ischemia on Reflex Magnitudes

Part A: Effect of aortic occlusion on the monosynaptic extensor reflex (filled circles) and polysynaptic reflex (unfilled circles) recorded as percent of control. The monosynaptic reflex was produced by gastrocnemius nerve stimulation and the amplitude of reflex discharge recorded from ventral root L_7 or S_1. The polysynaptic discharge was initiated by sural nerve stimulation and areas beneath the ventral discharge curve were measured planimetrically.

Part B: Effects of nitrogen administration on monosynaptic and polysynaptic reflexes. Stimulus and recording conditions are as in Part A. Note the longer time course of failure during hypoxia than during aortic occlusion.

Note that in both parts the polysynaptic reflex augments sooner and fails before the monosynaptic reflex.
(From Kirstien (44).

A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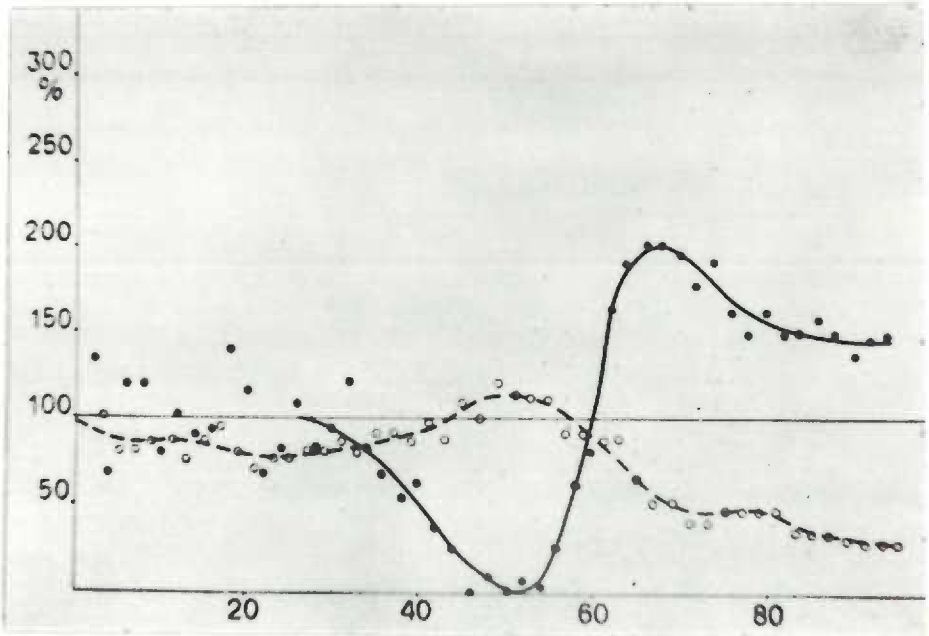
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80

speculate any further about possible sites or mechanisms of hypoxic reflex failure.

Previous to Kirstein's work, Van Harreveld (57,58) had reported that the flexion reflex survived longer than the monosynaptic reflex tension development induced by tendon tap. However, Kirstein's results clearly showed that monosynaptic reflex discharge outlasts polysynaptic reflex activity. These reflexes were produced by peripheral nerve stimulation. Since contractures were reported during hypoxia it is difficult to imagine how Van Harreveld was able to maintain any semblance of stable stimulating conditions. Kirstien, of course, would not have had these problems. Van Harreveld also noted that in cats previously made hypoxic, for 25-35 minutes, monosynaptic reflex responses returned during the ensuing two weeks of recovery, whereas flexion reflexes were depressed or absent. Even Van Harreveld concluded that in this respect the monosynaptic reflex is more resistant to hypoxia than polysynaptic reflexes. Both investigators suggested that perhaps internuncials were more susceptible than motor neurons to hypoxia; a conclusion born out from the histologic studies. Since the motor neurons are the final common path for both mono and polysynaptic reflexes, polysynaptic failure must be presynaptic in nature or at least pre motor neuron.

An attempt to further define the weakest element in hypoxic reflex failure requires information about spinal neuron function inside the cord. Gelfan (54) compared cord dorsum and ventral root potentials in spinal cord of cats made hypoxic by aortic occlusion.

Figure 6A contains cord dorsum potentials recorded during 38 minutes of ischemia. The longest lasting portion of this signal is the initial triphasic (pos-neg-pos) deflection due to the afferent volley. Since peripheral nerves are known to survive for long periods, this result is not surprising. The late long lasting positive deflection disappears after 2.5 minutes and is the first portion of the cord dorsum potential to disappear. Gelfan ascribes motor neuronal discharge as the cause of this p wave. More recent evidence indicates that this signal is due to primary afferent depolarization (17). Consequently, Gelfan must have been in error when he concluded that the motor neuron is the most susceptible element to hypoxia. The large negative deflection between the afferent volley and the p wave was observed to outlast motor neuronal reflex discharge (Part B). Gelfan believed this portion of the signal represented internuncial discharge. However, it is difficult on theoretical or practical grounds to differentiate with confidence between internuncial and motor neuronal discharge, or EPSP. Therefore, Gelfan's paper is not critical in determining the "Differential Vulnerability of Spinal Cord Structures to Hypoxia." Later investigators have abandoned the ambiguous cord dorsum potentials in favor of more direct observations of motor neuron and spinal neuron function.

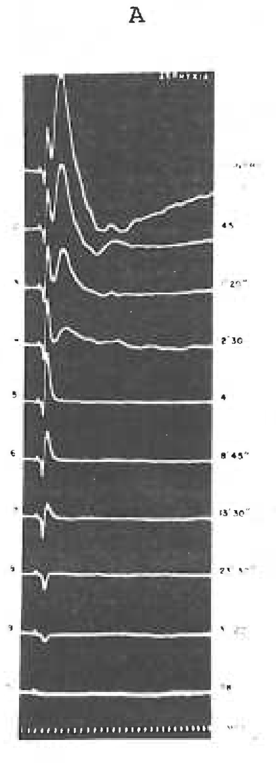
Another means of identifying activity in intraspinal neurons is to insert a micro-electrode into the motor neuron pool and thereby sample from a small anatomically distinct population of

FIGURE 6. Effects of Hypoxia on Spinal Cord Potentials

Part A: Cord dorsum potentials (negativity upwards) in response to dorsal root stimulation during 38 minutes of asphyxia. Note the persistence of the initial triphasic wave and relative sensitivity of the late positivity.

Part B: Effect of ischemia (aortic clamping) on spinal reflex potentials. Cord dorsum potentials (upper trace) and ventral root potentials (lower trace) reflexly induced by dorsal root stimulation. Note the disappearance of the ventral root response after 4 minutes and incomplete recovery after 54 minutes post ischemia. Cord dorsum potential seems more resistant.

[(From Gelfan and Tarlov (54)].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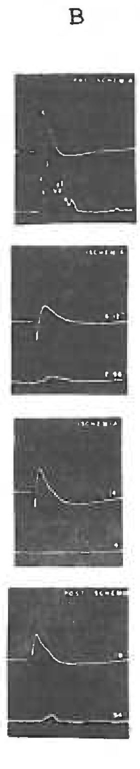
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54

neurons. This focal potential recording technique and analysis was described in an earlier section of this introduction.

Focal potential recordings by Brooks and Eccles (59) have been used to elucidate the site of hypoxic reflex failure. Figure 7A contains records of these focal potentials as the dorsal root induced reflex failed during hypoxia. The initial positive (downward) deflection of the afferent wave could be discerned throughout the whole period of hypoxia. The large late negative wave signaled the motor neuron discharge, and disappeared after 3.5 minutes. The records in subpart b of this figure show non-synaptic excitation of the motor neuron pool by antidromic (backward) propagation of discharge. The initial positive axonal signal outlasts the negative soma-dendritic signal as had been previously suggested. When both a and b of Part A in this figure are compared, one can see that after 4 minutes of hypoxia the orthodromic reflex discharge of motor neurons has failed, whereas the motor neuron somata were still excitable to antidromic invasion. Brooks and Eccles concluded that reflex failure occurred because the presynaptic afferent terminal fibers are most sensitive to hypoxia and fail to conduct the afferent volley to the synapse on the motor neuron.

More recent studies similar to the above more clearly distinguish between the afferent and efferent elements of the monosynaptic reflex arc (56). Figure 7B shows that during ventilation with 5% oxygen the biphasic afferent signal remained essentially unchanged

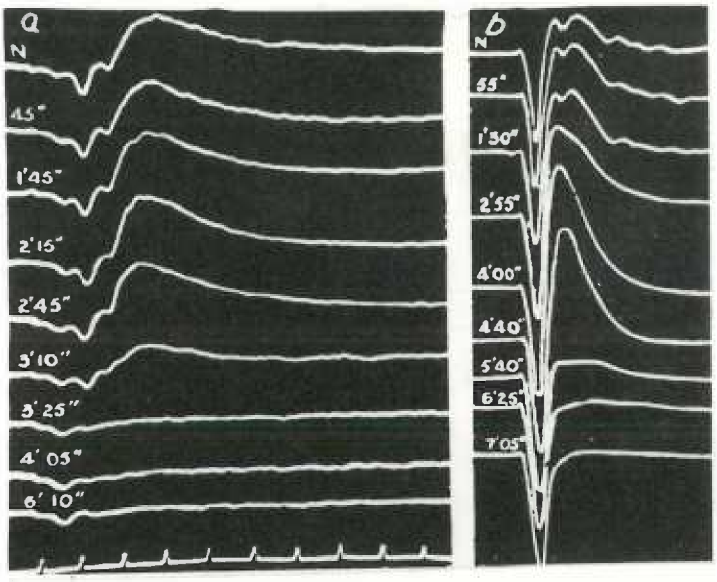
FIGURE 7. The Vulnerability of Presynaptic and Postsynaptic Structures to Hypoxia and Ischemia

Part A: Focal potential records in response to orthodromia (a) and antidromic (b) stimulation in the same cat during an episode of 100% nitrogen hypoxia. Note the disappearance of the late negativity in the orthodromic record at 3'25" which indicates loss of reflex motor neuron discharge. However, the antidromic focal potentials indicate that the motor neuron is still excitable at 4 minutes of hypoxia, negativity upwards.
(From Brooks and Eccles (59)

Part B: A recent focal potential study in the cat spinal cord. Records are taken serially from a trial in one preparation. a: control; b: 8'30"; c: 13'50"; d: 17'20"; e: 22"15"; g: 58'30"; h: 61'; i: 64'. Note the difference between the susceptibility of the presynaptic focal potential (initial triphasic wave under 5% O_2 and 100% N_2.
(From Eccles et al. (56). Negativity upwards.

Part C : A table comparing the survival times in minutes of various responses of the motor neuron as measured by intracellular recording from 17 cat lumbar motor neurons. The columns are ordered left to right in increasing means of survival time. Note that in 9 out of 11 motor neurons the antidromic spike outlasted the orthodromic (synaptic) spike. In all cases the initial segment spike outlasted the orthodromic (soma-dendritic) spike. In only three cases could EPSP's be detected after the soma failed to conduct an antidromic spike.
(From Collewijn and Van Harreveld (60)

A



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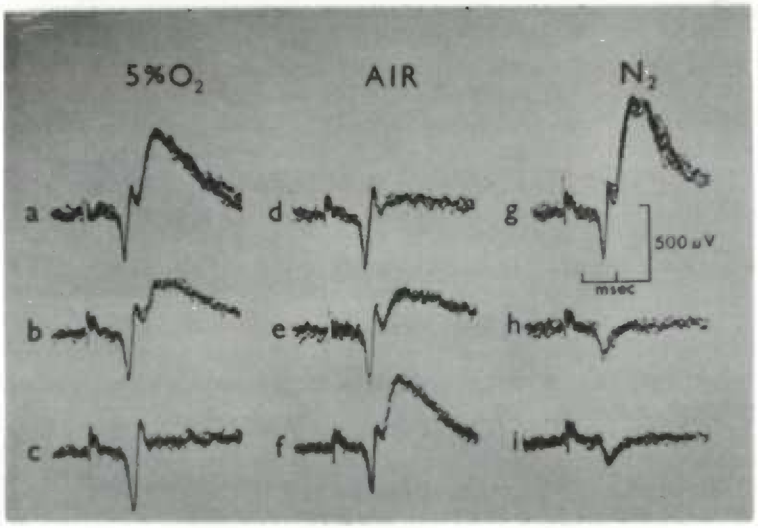

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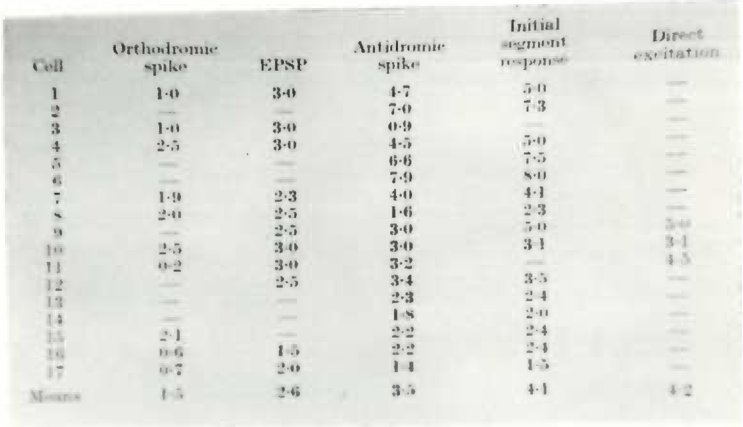


Cell	Orthodromic spike	EPSP	Antidromic spike	Initial segment response	Direct excitation
1	1·0	3·0	4·7	5·0	—
2	—	—	7·0	7·3	—
3	1·0	3·0	0·9	—	—
4	2·5	3·0	4·5	5·0	—
5	—	—	6·6	7·5	—
6	—	—	7·9	8·0	—
7	1·9	2·3	4·0	4·1	—
8	2·0	2·5	1·6	2·3	—
9	—	2·5	3·0	5·0	5·0
10	2·5	3·0	3·0	3·1	3·1
11	0·2	3·0	3·2	—	4·5
12	—	2·5	3·4	3·5	—
13	—	—	2·3	2·4	—
14	—	—	1·8	2·0	—
15	2·1	—	2·2	2·4	—
16	0·6	1·5	2·2	2·4	—
17	0·7	2·0	1·4	1·5	—
Means	1·5	2·6	3·5	4·1	4·2

while the postsynaptic response disappeared. However, during ventilation with N_2 the negative portion of the afferent signal decayed with the postsynaptic response. One might conclude from the 5% O_2 experiment that the failure was due to motor neuron inexcitability since the afferents appeared to be active even after the reflex failed. However, the N_2 experiment clearly showed that the afferents were sensitive to hypoxia and led the authors to conclude that failure was due to afferent terminal depolarization. In support of this conclusion, it was discovered by direct stimulation that the afferent terminal excitability slightly increased and then abruptly diminished to zero after 2 minutes of exposure to 5% oxygen. This observation is consistent with the concept that the terminals depolarized during hypoxia. Unfortunately, the authors of this paper did not provide a comparison of the temporal course of the afferent terminal excitability or reflex activity. The results are not clear though the authors favor presynaptic failure as the cause of reflex failure under hypoxia.

Kolmodin and Skoglund (61) first recorded intracellularly from spinal neurons of cats undergoing asphyxia. The resting membrane potential of the motor neurons was seen to decrease over a period of 2 minutes. Spike amplitude did not change until after this period when it finally, and rapidly decayed and the cell became inexcitable. They also showed recordings from internuncial neurons. Though these investigators did not tabulate their data, they report that orthodromic spikes were often blocked before antidromic spikes. In addition, they suggest that interneurons may be less sensitive than motor neurons to hypoxia.

Nelson and Frank (62) have used bipolar concentric electrodes to record resting transmembrane potentials from motor neurons of the cat. This study reports great technical difficulties as the onset of hypoxia causes blood pressure fluctuations and consequent electrode movement. Moving the electrode only a few microns alters or destroys the potential recording and makes interpretation of their work difficult. All their published records consistently contain movement artifacts and from little evidence they conclude that motor neurons are remarkably resistant to hypoxia. They apparently have observed motor neurons whose resting membrane potentials changed only 1-5 mv over a period of 2.5-3 minutes of asphyxia. Kolmodin and Skoglund had earlier reported "moderate depolarization" during the first minute, and rapid depolarization for some undefined period thereafter (61).

In a study which has not attracted much attention, Washizu (63) reported the results of intracellular recordings from isolated toad spinal cords. The orthodromic, antidromic, and directly stimulated motor neuron generated action potentials which underwent gradual reduction in amplitude over a period of 30 minutes during hypoxia. These are considerably longer survival times when compared to mammalian studies. Failure of synaptic transmission (orthodromic) was attributed both to a reduction in excitability of motor neurons as measured by direct stimulation, and to reduced excitatory synaptic potentials (EPSP). Washizu believes that the reduction in EPSP could arise from failure of the presynaptic fibers.

It is common characteristic of the intracellular work discussed so far that very little information is given concerning the number of experiments performed, reproducibility of results, or any quantification of parameters such as mean survival time, antidromic vs orthodromic survival, etc. One is left with the feeling that these experiments were exploratory and preliminary observations, as no clear hypotheses were proffered or tested and the mechanisms of failure only postulated.

It is refreshing, therefore, to discuss the recent work by Anthonie Van Harreveld and his collegues (60,64). First of all, Van Harreveld reported that the spinal grey matter in cats undergoing stagnant hypoxia slowly begins to develop a negative potential relative to the surface of the spinal cord (64). The focus of this negative potential appears to be intermediate between the dorsal and ventral horns. This asphyxial potential is attributed to depolarization of the "intraspinal part of the motor neurons" (dendrites) and primary afferent terminals.

Van Harreveld postulates two mechanisms by which hypoxia may affect reflex activity (41,60,65). Hypoxia causes (1) increased sodium ion permeability and/or (2) arrest of the ion pumps. Since dendrites and fine terminal branches of axons have a high surface area to volume ratio, these elements would be more rapidly affected than the somata or axons. By either mechanism hypoxia would result in the movement Na^+, Cl^- and water into the neuron with resultant decrease in the transmembrane resting potential (41). The cause of the postulated increase in sodium permeability is attributed to the release of a chemical substance perhaps glutamate. However, R. Eccles (56) has observed that the motor neuron membrane resistance does not change during hypoxia, an observation which casts doubt on the notion that hypoxia causes an increase in ionic permeability. Furthermore, Ito and Oshima (66) have obtained values for sodium permeability of spinal motor neurons of the cat. Their calculations indicated that the rate at which spinal motor neurons depolarize during hypoxia is about what one would expect from simple inhibition of active sodium transport. Consequently, there is no need to postulate the release of a depolarizing agent in spinal cord, but the mechanism of hypoxia-induced spreading depression in cerebral cortex may require such a postulate. Van Harreveld has evidence that intracellular Cl^- and extracellular impedance increase during asphyxia of the CNS which is consistent with this proposed mechanism of failure (41). The motor neuron would be excitable to antidromic invasion after orthodromic invasion has failed if failure is due to depolarization of the fine primary afferent terminals. Van Harreveld also remarks on the great technical difficulty under which his intracellular experiments were performed. In spite of this, he tabulates (Fig. 7C) his results so that the reader has an idea of the quantity of experiments and the reproducibility of results. Even though Van Harreveld fails to mention it, the mean survival times of his intracellularly monitored orthodromic responses to dorsal root stimulation (1.5 minutes) were considerably less than what he reported from ventral root recordings (2.5 - 4.5 minutes in an earlier paper (55)). Perhaps Van Harreveld's cells were partly damaged by impalement of the micro-electrode. In two out of ten cells the antidromic response failed before the orthodromic. However, one can apply the Wilcoxson's signed rank test to this data to test the hypothesis that there is no difference between the medians of the antidromic and orthodromic survival times. Since this test ($T=4.5$) rejects the null hypothesis at the 5% level it is not hard to be convinced by Van Harreveld's data that the motor neuron is less sensitive to asphyxia than the rest of the monosynaptic reflex path.

The weight of the evidence so far suggests:

1. The monosynaptic reflex is less sensitive than the polysynaptic reflex.

2. Motor neuron is the least sensitive element in the reflex path to hypoxia.

The postulated mechanism of reflex failure is that <u>hypoxia prevents active transport which results in passive depolarization of the neurons</u>. Depolarization would proceed most rapidly in fine terminal axon branches and distal dendrites (41,43,56,59,67,68).

The most recent publication in this field is also by Van Harreveld and his associates (69). The next logical step in the development of his concept of hypoxic reflex failure would be to examine the effects of hypoxia on internuncial neurons by intracellular recordings. Technically even more difficult than his work on motor neurons, this work is based on the results from only 19 interneurons. These cells become inexcitable to orthodromic and direct activation at about the same time during asphyxia. Furthermore, he was able to observe excitatory postsynaptic potentials after the cell failed to discharge in response to direct stimulation. The mean survival time for discharge in these cells (3 minutes) is longer than that for motor neurons (1.5 minutes) under similar conditions. In contrast to the conclusions from his motor neuron studies, Van Harreveld is forced to conclude that the principle cause of failure of synaptic activation in interneurons is due to the depolarization of the interneuron somata rather than the presynaptic axon terminals. However, it is possible to conceive that primary depolarization of the interneuron dendrites electrotonically depolarizes the interneuron soma. If dendritic depolarization is powerful enough it could result in postsynaptic failure before the presynaptic terminals had depolarized. One is still required to explain why the survival time of the internuncials is longer than Van Harreveld previously observed for impaled motor neurons.

If one accepts the conclusions of this paper, it is necessary to alter the interpretation of other literature in the field. Why do polysynaptic reflexes (involving internuncials) fail before monosynaptic if internuncials are less sensitive than motor neurons?

Is an internuncial membrane different from a motor neuron membrane? Are presynaptic terminals to the motor neuron more sensitive than those to the internuncials? The results from this paper make generalizations from monosynaptic studies quite difficult.

Summary and Conclusions - Part I

The only general agreement in the literature is that hypoxia causes reflex failure. Starting from here one can conclude that the motor neuron is the least susceptible element in the monosynaptic reflex pathway. Next, the balance of evidence indicates that internuncial paths (polysynaptic) may be more sensitive than monosynaptic pathways. If so, why are internuncials less sensitive than motor neurons? Could it be that the "weak sister" is the primary afferent neuron?

Concerning the mechanisms of hypoxic reflex failure, the most attractive hypothesis is as follows:

> Active ion pumps are inhibited when hypoxia reduces the supply of ATP necessary to their operation. The result is an uncompensated passive influx of Na^+ and efflux of K^+ which causes depolarization of the cell. This ion movement should have the greatest effect on portions of the neuron that have high surface area to volume ratios. These are the dendrites and fine terminal branches of the axons. Consistent with this mechanism is the observation by others that hypoxia monosynaptic reflex failure is presynaptic in origin - i.e. fine terminal branches.

However, there is one basic assumption made in all of the intracellular studies mentioned so far. This assumption is that the micro-electrode is sampling from near the region of motor neuron membrane participating in synaptic junctions with monosynaptic afferents, i.e., that monosynaptic afferents always make axo-somatic synapses. What if the stimulated afferents were synapsing on distal dendrites instead of motor neuron somata? Heneman (52,53) has shown electrophysiologically that mammalian motor neurons are indeed innervated on distal dendrites by monosynaptic afferents. These observations are substantiated by recent histologic evidence (19). These studies make it impossible to be certain that a given motor neuron has axo-somatic synapses from the monosynaptic afferents. Furthermore, a micro-electrode in the soma might not be sensitive to potential changes on distal dendrites. Therefore, reflex failure could occur in dendrites of the motor neurons before the afferent terminals fail. As a result the somata of motor neurons would still be invaded by antidromic action potentials after the reflex failed. This test of somal excitability does not test distal dendritic excitability. Thus antidromic excitability after reflex failure does not prove that the failure was pre-synaptic in origin. Consequently, the micro-electrode experiments on mammalian spinal cords cannot distinguish between presynaptic failure and postsynaptic failure with complete certainty. As a result the conclusions drawn by the above authors must be viewed with some caution.

INTRODUCTION

PART II - THE ENERGETICS OF ION METABOLISM IN NERVE TISSUE

ELECTROCHEMICAL GRADIENTS ACROSS CELL MEMBRANES

It is an invariable observation that ion concentration differences exist across all living cell membranes. Since electrical potential differences are concomitant with these ion differences, an electrochemical gradient is established across cell membranes.

Transport theories to explain this phenomenon abound in the literature of the past six decades. Donnan's (70) thermodynamic treatment of equilibria explains concentration gradients across cell membranes by merely passive, non-energy consuming principles. Bernstien (71) postulated that the resting membrane potential was the result of K^+ permeability and NaCl impermeability. Boyle and Conway (72) lent support to this theory by their observation in frog muscles of concentration differences for K^+ predicted by Donnan equilibrium, but definitely showed that the cell was also permeable to chloride ion. However, if Na^+ is also permeable, biological tissue would be unable to sustain the enormous osmotic pressure differences established by complete Donnan equilibrium. Unless these membranes become impermeable to water or electrolytes at some stage in their development, Donnan equilibrium would cause cell lysis [(73) p. 82].

Radioisotope studies, direct tissue analyses and ion specific electrodes are some of the techniques used to conclusively demonstrate that cells are permeable to both Na^+ and K^+ and that a constant movement of each of these ions exists in both directions through the cell membrane (74,75). Apparently a steady state (stationary state) electrochemical gradient is present rather than equilibrium (76,77).

The consequence of a steady state condition is that a continous expenditure of metabolic energy must prevail in order to maintain it. Since, in the steady state Na^+ is not in equilibrium, and Na^+ flux out of the cell (efflux) is against an electrochemical gradient, energy must be consumed in the maintenance of this flux. Although the mechanism of such a process is not known, metabolic energy linked movement of a solute is defined as active transport (78,79). Formalization based on the thermodynamics of irreversible processes has also provided criteria for defining active transport (80,81).

The purpose of the following sections is to present evidence that the maintenance of the steady state in nervous tissue is dependent upon the expenditure of metabolic energy.

STEADY STATE DEPENDENCE ON OXIDATIVE METABOLISM

Chemical Composition of the Nervous System

The chemical composition of the nervous system is as specialized as its function (82). Furthermore, it is quite heterogenous in composition, especially when white and grey matter are compared (83). The lipid content of brain is far greater than that of any tissue except adipose tissue (82). Yet, the central nervous system (CNS) contains little triglycerides or cholesterol esters (84). About 60% of the brain lipids, and those that are most specific to the nervous system, are found in myelin.

The CNS is relatively deficient in protein, containing only about 30% of the amount found in an equivalent weight of muscle. This is reflected somewhat by neuronal enzyme concentrations. For instance, mitochondrial concentration of cytochrome oxidase is about 60% of the concentration found in liver (85). Oxidative enzymes appear to be associated with mitochondria which are found in the somata, dendrites, terminal axon, and the nodes of myelinated axons (86). The CNS, unlike most tissues, contains little glycogen (83,87). This may explain neuron functional dependency upon moment-to-moment delivery of substrate by the blood since glycogen is a source of metabolic energy.

Energy Metabolism in the CNS

Lipid turnover by the CNS is relatively slow, and neuronal lipid content does not fluctuate with body weight changes (88). Protein energy metabolism is related to the amino acid metabolism. The mammalian brain contains amino acid concentrations between two to eight times the concentration in plasma, depending on the species observed (82).

Carbohydrate metabolism is not remarkably different from that of other tissues. All the representative pathways are present but the Embden-Myerhof pathway supports the most traffic. Glucose is the predominant substrate for energy metabolism in the CNS and under normal conditions, the brain respires exclusively at its expense (89).

Since the RQ of the CNS is near unity, it was once thought that glucose was solely oxidized by the Embden-Myerhof pathway. Radioisotope studies have shown that only about 60% of the glucose enters this pathway directly and the rest passes to intermediates such as amino acids which are in time oxidized to CO_2 and H_2O (89).

One of the major points of departure from simple glycolysis occurs in the Tricarboxylic acid cycle (TCA). Glutamic acid and glutamine, considered important metabolites because of their abundance in the CNS, may be synthesized from TCA intermediates. Furthermore, Terner (90) showed that K^+ depleted brain slices would recover initial K^+ values only when glutamate and glucose were both present; neither were effective lone. A second amino acid, gamma amino butyric (GABA), is also prominent in nervous tissues. The conversion of glutamate to GABA may be catalyzed by a transamination or directly by glutamic acid decarboxylase (GAD). This enzyme is found only in the mammalian CNS and largely in the grey matter (91).

Thus it can be seen that nervous tissue contains the necessary substances to carry out oxidative metabolism. It was shown in Part I that the functional properties of reflex activity are critically dependent upon continuous delivery of oxygen to the system. The next section will discuss the metabolic rates as measured by different parameters.

Parameters of Oxidative Metabolism: Glucose and Oxygen Consumption Heat, Production

Oxygen consumption of nervous tissue, the most oovious and most easily measured parameter of metabolic activity, has a long history of study. It has been reported (92) that Thunberg in 1904 first succeeded in detecting respiratory activity in isolated nerve.

Since then, observations have been made in other isolated segments of the central nervous system (93,94,95,96,97), homogenates (94,98), mitochondrial suspensions (98), in vivo (99), and in situ (100,101).

For adults in vivo, the CNS accounts for about 20% of the total body basal oxygen consumption (99). The oxygen consumption varies with the age of the individual, and the portion of the tissue studied (83,99). In vitro slices of cortex, medulla, and spinal cord respire with relative ratios of 100:34:12 (95). Grey matter is highest in oxygen consumption, whereas white matter and peripheral nerve are lowest (83). This would suggest a greater metabolic rate in the soma-dendritic portion of the neuron as opposed to the axon. In fact, on the basis of the distribution of enzyme activities, Lowry (102) considers the source of grey matter metabolism essentially dendrites.

The relative oxygen consumption of different tissues, different portions of nervous system, and different species have been tabulated from Tower (83). (See Table 1).

One problem in making comparisons between different authors is that different units are used. Oxygen consumption under standard conditions is often expressed as µl /mg dry wt/hour (equals QO_2), or ml/100 g wet wt/minute, but perhaps the most informative and most recent expression is µmoles/gram fresh tissue/hour.

Expression of oxygen consumptions per gram by nervous tissue is not the only way to relate metabolic rates. Comparisons of tissue on a per cell basis eliminates differences due to cell types and density. Expressed in this manner, neuronal QO_2 is about four times....

TABLE 1

Tissue	Total Nuclei per mm^3 X 10^3	O_2 Uptake ($\mu l/hr$) per mg fresh wt.	O_2 Uptake ($\mu l/hr$) per 10^6 nuclei
Cat			
Cerebral cortex....................	128	2.4	19
Corpus callosum....................	135	0.77	5.7
Cerebellar cortex..................	808	2.1	2.6
Dog			
Cerebral cortex....................	148	2.15	14.5
Corpus callosum....................	145	0.69	4.8
Cerebellar cortex..................	568	1.7	3.0
Man			
Cerebral cortex....................	131	1.9	14.5†
Corpus callosum....................	112		
Temporal lobe white................		0.75	6.7†
Cerebellar white...................	42	0.33	7.9
Medulloblastoma‡...................	1170	0.52	0.44
Astrocytoma‡.......................	210	0.19	0.91
Oligodendroglioma‡.................	298	1.4	4.7

[From Tower (83)]

.... that of liver parenchyma(83). An even better method might be comparison by cell surface area, since it will be pointed out later in this paper that surface area, ion movements, and metabolic rates may be interrelated. However, surface areas for irregularly shaped cells are difficult to calculate (103).

When examining the O_2 consumption of nervous tissues, it should be clear that the material is tissue - not just neurons. The neuroglia and other connective tissues are responsible for a considerable amount of respiration in the CNS and may be affected by conditions designed to affect neurons only (104). They may comprise as much as 70% to 80% of the total cellular population of the cerebral cortex (33). Comparison of cerebral cortical (neurons and glia) respiration with that of the corpus callosum (axons and glia) led Korey and Orchen (106) to postulate that the respiration of the cortex was 76% neuronal, of white matter 30% neuronal, and the respiration of the whole brain 66% neuronal. However, Hamberger (105), using the elegant microdissection techniques of Hyden found that glial metabolic rate was equivalent to or much greater than that of neurons. Enzyme activities per unit volume or unit dry weight were greater in glia than in neurons. Others (see Tower (83)) claim to have evidence that glia respire less on a per wet weight basis than cerebral cortical neurons but more than cerebellar cortical neurons. Furthermore, if evidence from neoplasms is applicable to the normal state, different kinds of neurolgia respire at different rates (see Table I). One can conclude, in spite of the technical difficulties involved, that neuroglia constitute a large part of CNS metabolism and must be recognized in discussions of nervous tissue oxygen uptakes. Apparently, no information is available that estimates the metabolic activity of connective tissue in peripheral nerve trunks and ganglia, but it is difficult to ignore the possibility of significant non-neuronal metabolism in these tissues.

What may be concluded from this section is that nervous tissue and neurons are highly active metabolically. That the nervous system is functionally dependent upon good oxygenation has already been established, but another substrate equally important to function remains to be discussed.

Glucose consumption is another commonly used parameter of metabolic rate. The nervous system is unusual in the fact that glucose is the only important exogenous substrate normally oxidized (89,99). The range of glucose consumption is dependent upon the pO_2. Nervous tissue, like others, shows the Pasteur effect. That is, when hypoxic tissue is exposed to oxygen the glucose consumption decreases. The mechanism of the Pasteur effect is not clear, but could be due to increased competition for ADP by the reactivation of oxidative phosphorylation (82). Naturally, unsuspected hypoxia in a CNS preparation could seriously confuse an estimation of oxidative energy consumption if metabolic rate was deduced from glucose consumption alone.

Six moles of O_2 stoichiometrically oxidize one mole of glucose to six moles of CO_2 (RQ=1). Glucose, however, may be used in vivo at a rate 16% greater than would be expected from the measured QO_2. Reasons accounting for this extra glucose uptake range from systematic error to requirements for synthesis of other substances not oxidized to CO_2 (99). But it is glucose that is important since TCA intermediates, and lactates are insufficient substrates for restoring the QO_2 of hypoglycemic slices or assuaging an insulin coma (99).

From a thermodynamic point of view, heat production is the most reliable parameter of energy utilization by nervous tissue. The initial measurements were performed in the early thirties and involved great technical difficulties. Further work was not again attempted until 1958 when better instrumentation enabled more sensitive measurements (107). One problem was to obtain large numbers of uniform diameter nerve fibers that could be synchronously activated and so that activity could be generated in all of the axonal membrane. This requires non-myelinated, small nerves in sufficient quantity to generate heat on the order of micro calories/sec. Furthermore, consideration must be given to heat capacity of the thermopile and heat sinks of tissue unassociated with activity.

The resting heat production of nerves has been studied (108). For frog sciatic at 20° C, 4.14×10^{-3} cal/g min. of heat is produced. During hypoxia the heat production declined over a period of three hours to 20% of its initial value (108). Readmission of oxygen rapidly resulted in heat production temporarily in excess of the resting oxygenated level, and this excess corresponded to 20% of the heat decrement during the hypoxia. Of greater interest is the heat production associated with activity but this will be discussed in later sections of this introduction.

Effect of Temperature on Oxidative Metabolism

It is a well known observation that chemical reactions increase in rate as the temperature increases, since the rate constants of all reactions are proportional to the absolute temperature (82). For enzyme systems, the rate of reaction approximately doubles with each ten degree centigrade rise in temperature. The Q_{10} of reactions is defined by the following expression and can be shown to be a function of the temperature (109).

$$\frac{\text{Rate @ T+10°C}}{\text{Rate @ T}}$$

For mammals, the Q_{10} of basal metabolism is about 2.3 (110). In amphibians, the Q_{10} for metabolic heat production ranges from 1.8 to 3.5 (111). Oxygen consumption as determined by the Fick principle for dog brain has been shown to be linearly proportional to temperature, and a simple calculation from the data means gives a Q_{10} equal to 3.3 (112). The passive transport of ions across cell membranes is much less sensitive to temperature than metabolic reactions. The Q_{10} for sodium influx has been measured at 1.2 to 1.6 for squid axons [(113), page 104]. Many other examples could be presented here, all in support of the general rule that metabolic rate increases with a rise in temperature. An interesting consequence of the small range of Q_{10} (2-3) of biological systems is that the overall activation energy must range from 15-20 k cal/mol.

The purpose of this review so far has been only to distill the voluminous information concerning CNS metabolism. Evidence was presented to indicate the high rate of metabolism by the CNS. It has been suggested that this high rate in nervous tissue is the result of considerable active transport (114).

Evidence Relating Active Transport and Oxidative Metabolism

Perhaps the most dramatic of experiments demonstrating active transport is the classical one of Ussing (78). In this experiment, Ussing devised a chamber in which a square of frog skin separated a pair of solutions identical in composition. He then proceeded to neutralize the inherent transepithelial potential by bucking voltages from electrodes in the solutions. Though this short circuit technique eliminated any electrochemical gradients, Na^+ continued to move in its usual direction. The inescapable conclusion is that there exists a sodium pump. That this sodium pump is metabolically dependent was proven by inhibition by low pH, increased PCO_2, hypoxia, and dinitrophenol. When the "outside" solution contained no Na^+, oxygen consumption fell by 35%.

Nerve cells and tissues of all kinds lose K^+ and gain Na^+ when exposed to hypoxia, or metabolic poisons (93,94,115). As expected, hypoxia results in a decrease of high energy phosphate compounds (ATP , arginine phosphate, etc.) in nerve, ganglia, and in CNS (83,93,94,116,90). Stewart (117) concludes from observations of the recovery processes, that the pH of mammalian nerve decreases under hypoxia. The fall in pH may be the result of lactate formation during anaerobic metabolism, but pH returns to normal within 30 minutes when the nerve is re-exposed to air. Analysis of the axoplasm of giant squid axon showed reduced levels of ATP and arginine after metabolic inhibition (14). Injection of arginine phosphate or ATP restored the efflux of Na^+ and influx of K^+ in the cyanide poisoned axon (14).

Whittam (79,118) has utilized portions of guinea pig cerebral cortex as a preparation in the study of ouabain. His basic assumption, for which there is considerable evidence [(113) page 305,(119),(120), (121)], is that ouabain specifically inhibits active ion transport. When ouabain was added to the incubation medium containing isolated portions of cerebral cortex, K^+ uptake by the tissue ceased and O_2 consumption fell 30-40%. If fresh cortex was incubated in Na^+ free Ringers, O_2 consumption fell but application of ouabain did not result in any further decrease of oxygen consumption. Similarly, K^+ uptake, which is depressed in isolated hypoxic cortex was not significantly decreased when ouabain was added to the hypoxic medium. These observations suggest that active ion transport requires and stimulates oxidative metabolism.

The discovery of membrane bound Na^+ and K^+ activated ATPases inhibited by ouabain also adds to the evidence that the sodium pump is metabolically driven (120). The pump is conceptualized in many ways (80,14), but the concepts usually involve an ion specific molecule (enzyme?) that results in the hydrolysis of ATP as Na^+ and K^+ ions are pumped against their electrochemical gradients. If the liberated ADP is rephosphorylated by the cytochrome system, oxygen is consumed. The ratio of K^+ transported to oxygen consumed has been calculated for several tissues. For mammalian cerebral cortex the value is 6-7.5 (118) and for frog muscle it is about 4. Na/O_2 has been estimated at 16-20 for frog skin, and 4-5 for rabbit nerve [(113) page 150].

It has been shown that the metabolic rate (O_2 consumption) of mitochondria and tissues is dependent on the amount of ADP present in the cell as a phosphate acceptor (122). Since most energy requiring processes in the cell result in the hydrolysis of ATP to ADP, we could imagine that the metabolic rate in cells is adjusted to the energy demands on the cell and is reflected in ADP generation. Removing Na^+ from the external environment of most tissues results in a decrease in the QO_2 and loss of K^+ from mammalian brain slices (94). Na^+ efflux decreases when squid giant axons are incubated in K^+ free media (14). These results suggest that Na^+ and K^+ active transport are linked and that the two ions are transported simultaneously. Thus, the absence of one ion in the medium would reduce active transport and therefore the energy demand on the cell. The reduced energy demand means less ADP is generated with a resultant fall in QO_2. If ouabain is used in the medium, the Na pump ceases and the QO_2 falls 40% (118). This suggests that nearly half of the resting respiration of CNS tissue is devoted to the maintenance of ion gradients (118,66). It is obvious then, that active ion transport plays a great role in the amount of metabolic activity exhibited by the nervous system. As will be shown in the following sections, nervous activity which involves ion movements should be expected to exert an influence on neuron metabolism.

METABOLIC ASPECTS OF NERVOUS ACTIVITY

Action Potentials: Hodgkin-Huxley Hypothesis

A considerable amount of background has been laid to this point in order to generate the concepts of the resting neuron physically, metabolically, and biochemically. Ion movements and energy metabolism are nearly inseparable subjects from the point of view of the nervous system. Generally, however, functional characteristics of neurons are not those of the static steady state that has been described above, but, in the normal condition, is one of constant dynamic activity. In order to appreciate the metabolic significance of neuronal activity it is first necessary to establish the mechanisms and events that occur during the passage and propagation of a single action potential by a neuron.

The giant squid axon, actually a syncitium formed from the fusion of hundreds of axons during ontogeny, has been the major preparation for the study of unmyelinated axon function {(14),page 11]. Its large diameter (up to 1 mm) allows insertion of small glass capillary tubes into the cytoplasm so that the inside of the axon may be compared electrically with the outside.

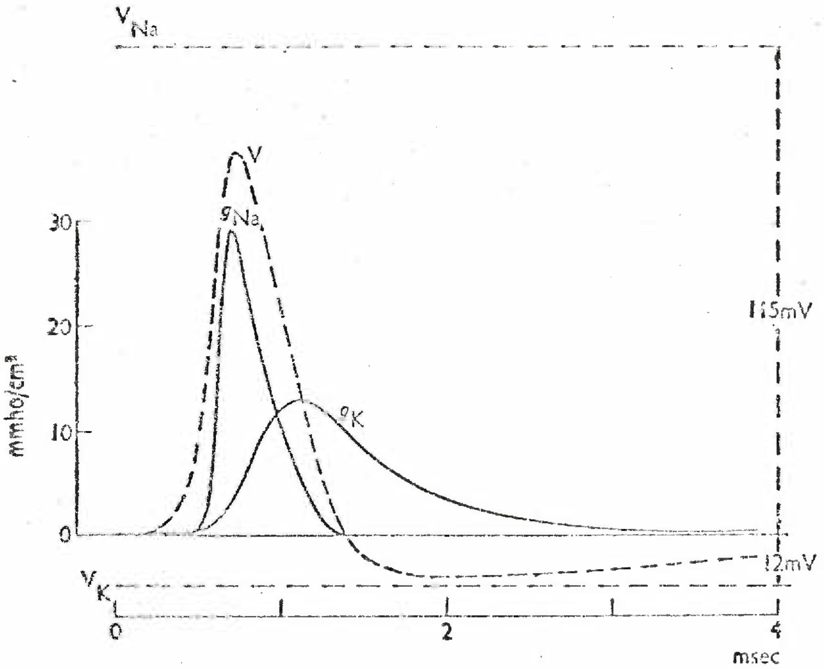
In a series of four papers, Hodgkin and Huxley, added to and summarized their observations on the electrochemical events that are associated with the generation of action potentials in giant squid axons (124,125,126,127). The last paper in the series developed the concepts into a mathematical description of the action potential based upon their estimations of the timing of events resulting in ion movement. These critical events require the steady state electrochemical gradients previously described for the resting neuron. In the resting state, Na^+ is relatively impermeable to the membrane and because of the sodium pump, it is prevented from reaching equilibrium in spite of a strong electrochemical gradient for movement to the cell. On the other hand, K^+ is relatively permeable, tending to diffuse through the membrane to reach electrochemical equilibrium. Because the inside of the axon contains a large quantity of non-diffusable anions (protein) this equilibrium results in an unequal distribution of K^+, higher inside than outside the axon, as predicted by Donnan (71). The concentration gradient established by K^+ is nearly equally opposed by an electrical gradient, which is oriented positive outside to inside. The resting membrane potential then, results predominantly from a K^+ equilibrium potential as expressed by the Goldmann equation (71).

Ion Movement During the Action Potential

In their landmark papers, Hodgkin and Huxley describe the electrical and ionic events that they observed during generation of an action potential in giant squid axon. In the first instant as an action potential is generated, the membrane permeability to Na^+ inexplicably increases a thousand-fold. Tantamount to opening the floodgates of a dam, Na^+ suddenly begins to move into the cell down its electrochemical gradient. Being much more permeable now than K^+, Na^+ begins to equilibrate. As the predominant diffusing ion, Na^+ carries its positive charges to the inside of the membrane and neutralizes the original resting membrane potential. In the first moments when the actual concentration changes are small, the Na^+ polarizes the membrane somewhat less than its equilibrium potential of 55 mv. The membrane is now charged negatively outside relative to inside. This reversal of membrane potential constitutes the rising phase of the action potential and occurs in about 0.5 msec. or less. But even as the peak of the action potential occurs, Na^+ permeability is decreasing and flux of Na^+ into the cell is reduced. Simultaneous with this process, K^+ permeability has increased and will cause another reversal of the membrane potential restoring the membrane potential once more to near that of the potassium equilibrium. Midway through the decline of the action potential, K^+ permeability begins to decrease and the efflux of K^+ from the axon abates. These events occur and end within one msec. Often the K^+ permeability does not return to its original state until after the membrane potential over shoots the resting level, resulting in an <u>after hyperpolarization</u> that decays over a period of a few msec. Fig. 8 is an illustration of the theoretical time courses of the ion permeability changes that develop. Actual observations agree well with this theoretical simulation.

At this point it is obvious that the axon has gained Na^+ and lost K^+. If the electrical capacitance of the membrane is known, the amount of ion movement required to charge the members to the peak action potential amplitude may be calculated. Assuming the capacitance of a giant squid axon to be one microfarad, Hodgkin [(14), page 44] estimated it would require 0.12×10^{-12} moles Na^+ to charge the membrane 120 mv. This is about 1/3 of the actual observed ion movement. For an axon 500 μ in diameter, the movement of 4×10^{-12} moles/cm^2 into the axon causes a negligibly small change in intracellular concentrations. It might be imagined, though, that with very fine fibers of small diameter, the movement of ions following

FIGURE 8. Theoretical solution for propagated action potential and conductances at 18.5° C. (From Hodgkin and Huxley (127)).



V_{Na}
V
g_{Na}
30
20
10
0
mmho/cm²
g_K
115mV
12mV
V_K
0
2
4
msec

a train of impulses could be sufficiently large to significantly change the axoplasmic concentration of these ions. If excitable tissue is to maintain its excitability over a lifetime, then a redistribution of ions must occur. That is, the initial conditions prior to the action potential must be restored. This would require metabolically dependent, energy consuming ion transport.

Evidence Relating Nervous Activity and Energy Metabolism

There exists a large quantity of literature discussing biochemistry and relating it to neuronal function. Structure-function relationships, especially with regard to membrane structures, will not be discussed here. The goal of the section is to present a few papers which best describe metabolic changes resulting from nervous activity, especially pertaining to the energy cost of nervous activity.

Parameters of Energy Costs During Activity in Nerves

A number of nerve preparations have been used to attempt an exposition of metabolism and neuron function. A nerve, composed of bundles of axons, is easy to isolate, functionally survives for long periods, and is a convenient preparation in which to monitor axon behavior. Consequently, this preparation has the longest history of study in which metabolism and nervous tissue function are compared.

Certain fundamental observations of axon function and, by extrapolation neuron function have been described. First, resting nerves consume oxygen and increase their oxygen consumption when caused to conduct action potentials (127). Secondly, nerves evolve heat, and increase their heat production when activated (107). Lastly, nerve function is susceptible to metabolic inhibition [(14), page 73]. Metabolic inhibition inevitably results in failure of axon excitability.

Oxygen Consumption

A comparative study by E.B. Wright (49) on the effects of O_2 lack on peripheral nerve suggest that the survival time for action potential generation is inversely related to the normal rate of O_2 uptake. Resting O_2 uptake by mammalian nerves is considerably greater (200 cc/gram/hour) than that of amphibians (frog 50 cc/gram/hour) and invertebrates (crayfish 13 cc/gram/hour). Under nitrogen, mammalian action potentials disappear within about 30 minutes, whereas frog nerve survives 170 minutes and crayfish in excess of 540 minutes. Furthermore, increases in temperature reduce the survival time of frog nerve. If one assumes a linear relationship between temperature and survival time, it is possible to estimate from Figure 3 of Wright's paper, a Q_{10} of 2.4 between 25 and 35° C.

Mammalian nerves were asphyxiated at 38° C, frog nerves at 25° C, and crayfish at 19° C. Thus the comparative survival times also correlate inversely with temperature, as well as O_2 consumption values. The only values given where comparison is possible at similar temperatures show the survival time of frog nerve at 38° C to be only about twice that of mammalian nerve and the O_2 consumption of frog nerve at 24° C to be greater than that of crayfish at 24° C and less than lobster at 19°C. However, the heterogeneity of nerve structure (myelinated vs unmyelinated), and the different numbers of fibers per nerve make it difficult to draw meaningful species comparisons from Wright's data. One may conclude that the susceptibility of function to hypoxia is related to temperature and therefore to metabolic rate.

Brink et al. (128) using the then new technique of polarography for measurements of dissolved oxygen, described the kinetics of O_2 consumption during stimulation of frog sciatic nerve. They noted that the O_2 consumption increased rapidly in a logarithmic manner at the onset of stimulation to some steady state value, and decreased less rapidly in the same fashion at the end of stimulation. With stimulation at 15-100 times per second, they also observed that the steady state rate of O_2 consumption was proportional to the stimulus frequency. However, the extra oxygen consumed per nerve impulse was greater the higher the frequency and the shorter the train of impulses. This last result is hard to understand even though Brink et al. explain this as characteristic of the kinetics of the chemical reactions involved and not a reflection of different energy requirements per impulse. They also confirmed observations by others (129) that O_2 consumption can be inhibited completely by azide, but that dilute solutions (0.2 M) only inhibit the extra oxygen uptake associated with activity and not the oxygen consumed at rest. Though the poisoned nerve remained excitable for up to 50 hours, the time for 50% reduction in the amplitude of the action potential was significantly less than in control unpoisoned nerves. Their conclusion is that the extra O_2 consumption associated with activity is necessary to maintain excitability. However, the implication from this study that dilute azide inhibits the Na pump metabolism only is not borre out by direct measurements of ion movement (130).

The O_2 consumption measured by Brink et al. is remarkably similar to prior heat production measurements (assume 1 cc O_2=5 cal) for both resting conditions and different rates of action potential generations (131). Thus, they believe that O_2 consumption may be a good measure of the energy requirements of nervous activity. The O_2 consumption during activity appears to be distinct from that of the resting cell since changes in the resting respiration have no effect upon the "extra O_2 uptake" which is differentially susceptible to dilute azide. Since methyl floroacetate has been reported to inhibit resting QO_2 but not the increase in oxygen consumption with activity (129), the conclusion of Brink et al. is that the metabolic support for nervous activity is via a metabolic pathway somehow distinct from the metabolic pathway utilized by resting nerve. Their results certainly imply a two compartment system, but the nature and location of these compartments is not known.

Heat Production During Action Potential Generation

Using crab nerves, A.V. Hill was able to detect the evolution of heat as the result of action potentials evoked in his preparation. He postulated that the source of this heat was chemical processes which caused the recovery to the initial conditions in the axon.

Recently, more refined measurements by Hill has enabled him to detect a small transient initial heat change associated with the action potential itself (107). The initial heat is diphasic; first a positive heat (evolution) is followed by a negative heat (absorption) of slightly less magnitude. Thus, Hill was able to detect the heat changes associated with the action potential (initial diphasic heat) and recovery processes (later positive heat). When the nerve was stimulated maximally the rate of recovery heat production was twice the rate characteristic of the resting level. This suggested to Hill that 50% of the maximal energy output of the nerve was required simply to maintain it in a state of readiness (107). The reader will recognize this value as one derived independently by Whittam (118) from oxygen consumption studies.

The Effect of Metabolic Inhibitors

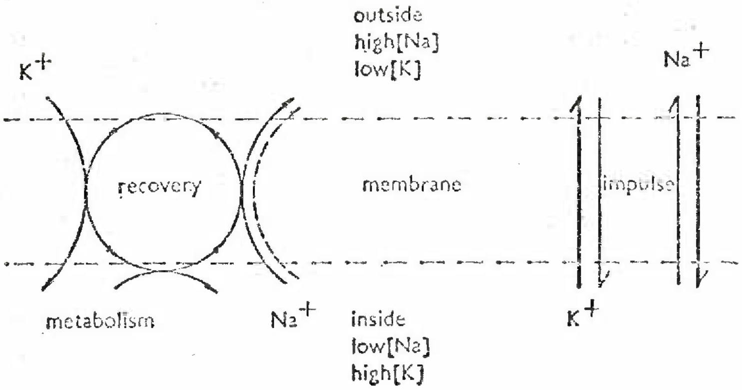
One simple method for assessing the importance of metabolism is to deprive the nerve of the benefits of metabolism (uncoupling) or by inhibiting metabolism altogether. Application of dinitrophenol (an uncoupler), CN, hypoxia, and iodacetate may fail to stop the generation of action potentials for 30-300 minutes depending on the source of the nerve [(14), page 73]. Eventually, however, the compound action potential does decrease and finally fails completely in the presence of the inhibitors (14). This change is thought by most investigators to be a result of resting membrane potential changes and not inhibition of the action potential generating mechanism. With metabolic inhibition membrane potential depolarization would result from the accumulation of Na^+ and loss of K^+ by uncompensated diffusional leakage, since the active ion pumps depend upon metabolically derived high energy intermediates.

In an earlier section of this thesis mention was made of the experiments by Hodgkin's group at Cambridge where injection of high energy phosphate compounds into metabolically poisoned squid axons restored sodium efflux. As Brink et al. had previously hypothesized, the Cambridge group proposed two separate mechanisms involved in ion movements. A simple scheme summarizing their concepts of ion metabolism related to neuron function is presented in Fig. 9. The energy source for the action potential is considered to be the free energy in the electrochemical gradients that normally exist in the resting steady state. A metabolically linked sodium-potassium pump restores the resting state after the perturbations of nervous activity. Therefore, the action potential generation mechanism is not directly dependent upon metabolism because only the recovery processes are metabolically linked.

There is a report by others that cyanide and dinitrophenol inhibit the action potential generating mechanism in frog nerves (132). Though these inhibitors did not have the same effect on squid axon it does not necessarily indicate that the action potential mechanism is metabolically linked in frog. Cyanide and dinitrophenol, in this case, may be nonspecific inhibitors acting on more

FIGURE 9. Diagram illustrating differences in "active" and "passive" transport of ions in the giant axon of the squid.

Pump	Spike Mechanism
Works uphill	Downhill
Recovery	Spike
Maximum rate: ca. 50 pmol/cm^2/sec	Maximum rate:ca.20,000 pmol/cm^2/sec
Inhibited by DNP, etc.	Not inhibited
High Q_{10}	Low Q_{10}
Not affected by membrane potential	Large effect
Not affected by calcium ions	Large effect


outside
high[Na]
low[K]
K+
Na+
recovery
membrane
impulse
metabolism
Na+
K+
inside
low[Na]
high[K]

than metabolism alone.

Phosphate Metabolism in Nerves During Activity

An interesting and logical sequel to the experiments of Brink et al. was done by Cheng (133). He measured the concentration of high energy phosphate (∿ P) compounds in frog nerve before recovery from hypoxia, or azide poisoning. As would be expected from the above discussion, these experimental conditions resulted in a decrease in ATP and creatine phosphate (CrP) and an increase in ADP. The concentration of these substances return to nearly initial values four hours after the experimental period. No measurements of inorganic phosphate (Pi) were done, however. Cheng does not describe the physiologic responses to stimulus (50/sec for 2 hours) except for the statement that the compound action potential disappears under nitrogen sometime during the two hour experimental period. In addition, he does not report an experimental temperature. It would be possible to assume room temperature, about 25°C, if Wright (49) had not observed the average survival time of frog nerve at this temperature to be almost three hours.

What is most interesting, however, is Cheng's attempt to correlate phosphate changes with oxygen consumption as measured by Brink et al. (128) and ion shifts as measured by Asano and Hurlbut (130). If the amount of oxygen consumed during activity, the amount of sodium accumulation, and the quantity of ∿ P hydrolyzed is known at the moment activity ceases, one can set up an energy balance table. (See Table 2). Cheng does this in terms of micromoles of high energy phosphates ($\sim P$). High energy phosphate consumption is calculated from QO_2 ($P/O=3$, lactate $(\frac{2\ \text{lactate}}{3\ \text{ATP}})$ and total change in measure $\sim P$ compounds, ($\Sigma\sim P=2\,[ATP]+[ADP]+[CrP]$). The energy "debt" after stimulation is the accumulated sodium ($Na/P=1$). Presumably this correlation could be most illuminating with respect to energy requirements, and utilization during nervous activity (see Table 3A).

Unfortunately, Cheng uses a value for oxygen consumed during the stimulus period which Brink et al. (128) derives to indicate rate of oxygen consumption. The proper value for oxygen consumed in excess of resting rate ("extra") during the stimulus period can be best calculated from Brink's data by performing the following integration: $QO_2\ \text{stim} = \int_0^2 RSS\,(1-e^{\frac{-t}{\alpha}})dt$

> Where QO_2 stim is the oxygen consumed during stimulation, RSS is the steady state or maximal rate of consumption (the value by Cheng), t is time (hours), α is time constant for the rise of oxygen consumption to the steady state value.

Evaluating the integral for two hours results in the value of 1.49 µmoles O_2/g 2 hr. during stimulation and before recovery. The value of total oxygen consumption including recovery is 1.76 µ moles O_2/g 2 hr. This would indicate that the nerve is only 85% recovered during the 20 msec. intervals between stimuli (50/sec). Cheng, however, does not take into account the implications of Brink's work and concludes that the total debt (sodium accumulation) at the end of stimulation is equivalent to 10 μ moles ∿P. Assuming a P/O of 3, this would require 1.7 μ moles O_2 to be consumed during recovery when one would calculate 0.27 μ moles O_2 from the data of Brink et al. Since Brink's oxygen consumption data compares almost precisely with heat production studies, one must conclude that it is Cheng's estimate of the energy debts that are in error. It is possible, however, to rework Cheng's values by assuming that three moles of sodium are transported by one mole of ∿ P (Na/P=3). This would result in a total energy debt of 3.3 μ m ∿ P, equivalent to 0.55 μ mole of O_2 which is much nearer the recovery value calculated from Brink et al (138). The total energy cost in terms of ∿ P is 13.2 μ m/g 2 hours. This is equivalent to a total extra oxygen consumption of 2.16 μ moles which is not considerably different from the 1.76 μ moles O_2/g 2 hours calculated from Brink et al. But when energy costs are estimated from azide poisoned nerves (extra oxygen consumption assumed to equal zero) the value of 8.2 μ moles ∿ P/g (1.32 mm/g) is 20% less than would be expected from the inhibited values. Since Cheng did not measure O_2 consumption, one cannot be certain that all of the extra O_2 consumption during activity was inhibited. However, Cheng's ∿ P data can, by manipulation, roughly be compared within ± 20% of what one could calculate from the oxygen consumption data of Brink et al. and this must be considered a good reasonable approximation considering the technical problems in measuring such low metabolic rates.

TABLE II

Retabulation from Corrected Values of Cheng(133)

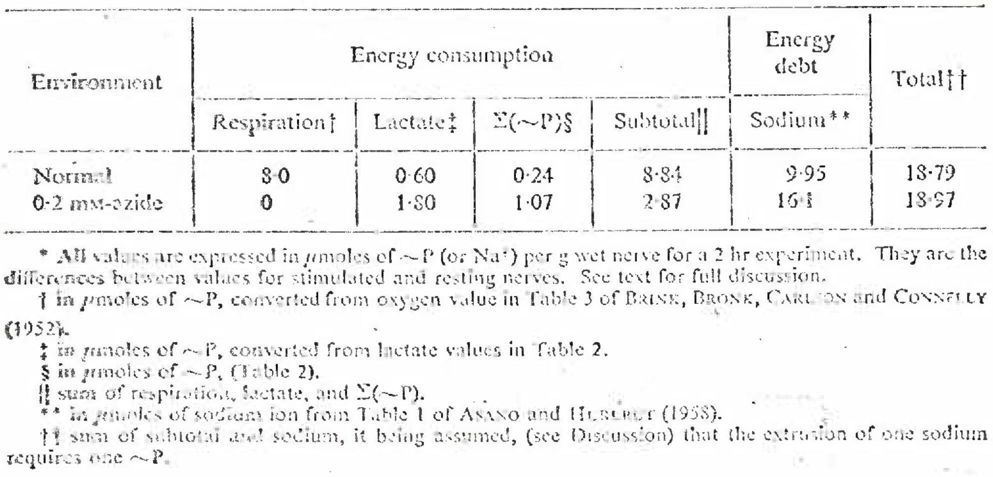
Environment	Energy Consumption				Energy Debt	Total
	Respiration	Lactate	Σ∿P	Subtotal	Sodium**	
Normal	9.0	0.60	0.24	9.95	3.3	13.2
0.2m Azide	0.0	1.80	1.07	2.87	5.3	8.2
Equivalent ∿P from O_2 Consumption*				9.0	1.6	10.6

All values µmoles ∿P per g wet wt. per 2 hrs.

* From Brink et al. (128) assume P/O=3

** Equivalent ∿P assuming Na/P=3

Cheng's Original Table (133)



Environment	Energy consumption				Energy debt	Total††
	Respiration†	Lactate‡	$\Sigma(\sim P)$§	Subtotal‖	Sodium**	
Normal	8·0	0·60	0·24	8·84	9·95	18·79
0·2 mM-azide	0	1·80	1·07	2·87	16·1	18·97

* All values are expressed in µmoles of ∼P (or Na^+) per g wet nerve for a 2 hr experiment. They are the differences between values for stimulated and resting nerves. See text for full discussion.

† in µmoles of ∼P, converted from oxygen value in Table 3 of Brink, Bronk, Carlson and Connelly (1952).

‡ in µmoles of ∼P, converted from lactate values in Table 2.

§ in µmoles of ∼P, (Table 2).

‖ sum of respiration, lactate, and $\Sigma(\sim P)$.

** in µmoles of sodium ion from Table 1 of Asano and Hurlbert (1958).

†† sum of subtotal and sodium, it being assumed, (see Discussion) that the extrusion of one sodium requires one ∼P.

Asano and Hurlbut attempted to estimate the energy cost of nervous activity from their observations of ion movements. However, they use 1000 cal/mole as the free energy of hydrolysis of ATP which in fact is the standard free energy. Because of dilution the standard free energy is about half the actual biological value. Thus, their energy calculations are too low by about 40%, and correcting this error increases considerably the apparent quantity of the energy reserves that they calculate.

Several years following Cheng's paper, Baker (134) published a similar but more comprehensive study of the phosphorous metabolism of crab (Maia) nerve. This preparation also has received the attention of A. V. Hill's group in heat production studies (107). The rationale for using this preparation is that the nerve, unlike frog sciatic, is completely unmyelinated. Presumably the numerous smaller diameter fibers and the greater surface area involved in action potential generation because the fibers are unmyelinated would result in larger and more easily detected energy changes. Baker's purpose was to demonstrate functionally that the sodium pump is, in fact, the ATP'ase described in crab nerve homogenates by Skou (120). By measuring inorganic phosphate (Pi) changes under various experimental conditions and including measurements of ion shifts and oxygen consumption, he could calculate some of the energy costs of nervous activity. Baker found that Na^+ accumulates in the nerve when exposed to K^+ free media, ouabain, cyanide, and stimulation (30/sec for 10 min). The Pi concentration of normal nerve increases 78% when stimulated, but does not increase at all if the active nerve is treated with ouabain. Baker concluded that the release of Pi from high energy phosphate compounds is triggered by an increase in internal Na concentration. Ouabain acts as a mixed inhibitor, decreasing both velocity and affinity of the sodium pump for K^+. By estimating the rate of breakdown of energy rich phosphates, Baker found that the rate of breakdown is proportional to the square of the Pi concentration. This means that relatively small increases in Pi result from greater rates of utilization and synthesis of high energy phosphates.

Baker's experimental results and his theoretical calculations can be applied to other studies if one is allowed to manipulate his data. Assume that the rate of breakdown of high energy phosphates normally equals the rate of synthesis (steady state). If cyanide is assumed to prevent resynthesis of $\sim$ P, then the Pi production of cyanide poisoned nerves is the result of breakdown of high energy phosphates, which in the steady state would be equal to the rate of synthesis. At rest, cyanide poisoned nerves produce 12.5 μ g Pi/g min and this rate increases with stimulation (10 min @ 30/sec) to 31 μ g Pi/g min (at 16° C). The oxygen consumption data collected by Baker can be converted to phosphate values (assuming P/O of 3 and Q_{10}=3) at rest of 12.4 μ g Pi/g min and during activity 32.8 μ g Pi/g min. These oxygen consumption values can be made equivalent (assume 1 cc=5 cal) to heat production values of 1.3 x 10^{-4} cal/g sec at rest and

2.6×10^{-4} cal/g sec during activity. Beresina and Feng (135) observed 1.9×10^{-4} cal/g sec for resting heat production. They did no measurements of heat production under the same stimulus conditions as those used by Baker. Consequently, Baker's experiments correlate well with various methods of measuring metabolic demands of nervous activity. However, this remarkable paper also contains an estimation of the energy cost of active transport. [See also (137)].

If ouabain is assumed to inhibit only the sodium pump mechanism, the Pi production in a cyanide-ouabain preparation should reflect the breakdown of high energy phosphates <u>not</u> associated with active ion transport. The difference between resting cyanide and cyanide-ouabain Pi production (12.5-3.2 μ g Pi/g min) is about 9 μ g Pi/g min. This represents the rate of high energy phosphate hydrolysis by the sodium pump under resting conditions and appears to be about 75% of the total resting rate of hydrolysis previously calculated. Again assuming the normal resting nerve is the steady state, it follows that the sodium influx during ouabain inhibition should be equal to the normal rate of sodium efflux by the sodium pump. The observed value is 0.78 μ mole Na/g/min. This value divided by the rate of Pi release due to sodium pump activity (0.78/0.29) gives a Na/P ratio of roughly 2.7. There is evidence that for other tissues the normal Na/P ratio equals three [(113), page 150].

It would be interesting to compare Baker's results with a theoretical estimation of the energy parameters of his system. From calculations in the appendix, the theoretical electrochemical gradient appears to be about 145 mv representing a Δ G=3340 cal/mole. The calculated free energy of hydrolysis of ATP at physiological concentrations is -11,600 cal/mole (136). Assuming a Na/P=3, the pumping efficiency of crab nerve approaches 88%.

These results are only approximations, and it is likely that the calculated values of metabolic acitivity from Pi production are underestimates. The assumption that cyanide inhibition on blocks resynthesis $\sim$ P denies any anaerobic energy metabolism. Such phosphate synthesis would not be reflected in oxygen consumption and would allow close correlation of oxygen uptake in normal nerves with Pi production in cyanide poisoned nerves. This would also explain in part the difference between the derived resting heat production of 1.3 x 10^{-4} cal/g min and the observed value of 1.9 x 10^{-4} cal/g min (135). The underestimate of phosphate metabolism also means that the efficiency would be lower than the theoretically derived value of 88%. Lastly, Baker's stimulus conditions make it impossible, with the exception of one experiment, to quantitate metabolism with a precisely known amount of nervous activity. The exception is the observed value of 1.6 p moles Na/g x 10^{-4}, which moves into the cell with each impulse. Given that the total surface area of the C fibers in crab nerve is 10^4 cm^2, Baker calculates that the amount of Na influx is 1.6 pm/cm^2/impulse.

A similar attempt has been made to estimate the energy cost of nervous activity on a standard mammalian nerve preparation. Using rabbit vagus nerve, values of 50 μ cal/g impulse have been observed and attributed to the C fiber population (138). Ritchie (139) converted his oxygen consumption data to heat production and calculated 100 μ cal/g impulse. The calculated free energy for sodium pumping is 2400 cal/mole. When energy requirements are calculated from observed ion movements (6000 p mole/g impulse or 1 p mole/cm^2 impulse) the minimum energy required per impulse is 14.5 μ cal/g. The observed O_2 uptake per impulse (1354 p mole/g) represents about 150 μ cal of energy, sufficient to supply a sodium pump at a rather low efficiency. The calculated $K/O_2=5$ suggests that the K/P ratio is probably near unity, or about 1/3 of what Baker observed in crab nerve. Since the resting K^+ efflux is 0.590 μ mole/g min in the rabbit vagus and the resting QO_2 is 0.0924 μ mole/g min, nearly all of the energy metabolism of the nerve (92%) is related to the maintenance of ion gradients.

When the "oxygen cost" of a single impulse in a single fiber C fiber is compared with that of a single frog myelinated fiber some startling results are revealed. Assuming 10^3 fibers @ 3.5 mg/cm in the frog nerve, the calculated O_2 per impulse cost for 1 cm of excited nerve is 2.8×10^{-7} moles (139). According to Ritchie (139) there are about 80,000 nonmyelinated C fibers in the rabbit vagus nerve with a linear weight of 1.8 mg/cm. The calculated oxygen cost per impulse is about 2.7×10^{-7} moles, or about the same in both fiber types. Hill has also shown that the initial heat production per cm^2 of nodal area in frog myelinated nerve is about 1000 times that observed for nonmyelinated fibers (139). These results suggest to Ritchie that the large surface area of nonmyelinated fibers in some way has been compensated for by energy conserving mechanisms. Furthermore, Hill (107) suggests that more total membrane than that of the nodes is involved in the nervous activity of a myelinated fiber.

Metabolic Aspects of Activity in Ganglia and the CNS

So far this discussion has only dealt with nerves or the axonal portions of neurons. Attempts at metabolic study in nervous tissues that contain cell bodies and synaptic connections are much more difficult to interpret because of the complexity of the tissue and the inability to quantitate nervous activity. The crayfish stretch receptor, axon, and cell body have been investigated by microfluorometric techniques, as have frog dorsal root ganglion cells and rat cerebral cortex (123,140,141). By observing the fluorescence of DPNH, one can monitor changes in metabolic activity in the tissue (123). Increases in metabolic or neuronal activity cause an oxidation of DPNH whereas metabolic inhibitors or anoxia generally result in reduction.

The isolated mammalian sympathetic ganglion has been studied by Larrabee and his co-workers (96,142). The oxygen and glucose consumption of this tissue is about ten times that of nerve trunks under similar conditions. Stimulation resulted in an increase in glucose uptake, lactate production, and oxygen consumption proportional to the stimulus frequency (40% at 15/sec). Lack of oxygen or glucose result in a loss of postganglionic response. The effect of hypoglycemia on the response is hastened if the preganglionic fibers are stimulated repetitively for a short time at a frequency that would not have altered the response in glucose. However, the functional failure under hypoglycemia is seen when oxygen consumption changes or ion shifts are small. On this basis it has been postulated that glucose is necessary to provide intermediates for the action potential generating mechanism, as well as maintain energy supply to the sodium pump (143). Stimulation and interference with metabolism by dinitrophenol result in loss of K^+ by the tissue. With metabolic inhibition, the postganglionic response may be lost when only 10-15% of the ganglionic potassium is lost. It has been suggested that failure of the response occurs in the fine terminal arborizations of the preganglionic axons, where changes in K^+ concentration sufficient to depolarize the endings would not be reflected in total ganglionic K^+ changes. Thus the presence of cell bodies and synaptic transmission does not alter the basic responses seen in nerves to increases in metabolic demand by activity or the changes associated with metabolic inhibition.

The brain, a target of many metabolic studies, is the most difficult of the nervous tissues with which to compare metabolism and function quantitatively. Attempts to assess the metabolic cost of "thinking" by providing subjects with mental tasks have failed to demonstrate any change in brain metabolism (99). (No guarantee exists that nervous activity increases with "mental exercise".) No consistent differences are observed in brain metabolism between sleeping and awake subjects or during EEG synchronization and arousal of perfused cat brains (101). On the other hand, cortical slices increase substrate consumption and lose potassium when stimulated on wire grids, even though electrical responses cannot be detected (93). Evidence of increased metabolism is seen in convulsing subjects and in the brains of mammals subjected to electroshock (145). It appears that to date, cortical slices, even when capable of responding electrically to stimuli (144), are so complex neurophysiologically and so heterogenous cellularly that it is impossible to control activation and measure the response with enough accuracy to relate metabolism quantitatively to nervous activity.

The spinal cord, it will be remembered from Part I, is well defined neurophysiologically. Distinct pathways can be activated in a controlled manner and motor neuronal discharge measured semi-quantitatively. Furthermore, the functional characteristics of each element in a reflex pathway can be monitored. Unfortunately metabolic studies on this tissue are scarce. The effects of hypoxia have already been discussed, but one group with experience in spinal hypoxia have extended their observations on motor neuron function by the ionophoretic injection of Na and azide (66). Ito and Oshima (66) demonstrated that the efflux of Na injected into spinal motor neurons was independent of the membrane potential but dependent upon an azide sensitive extrusion mechanism. Extending their conclusions by observation of the kinetics of injected ion movements, they found that the sodium pump was considerably more active in mammals (at 37° C) than frogs. The rate constant is 8-10 times greater than the sodium pump of amphibians even when corrected for temperatures differences (Q_{10}=3). This represents a Na pump rate of about 77 pm/cm^2 sec. Using accepted values for the concentration gradients the free energy for sodium transport is about 3500 cal/mole. Thus the minimum energy requirements for resting spinal motor neurons should be (77 pm/cm^2/sec x 3500 cal/mole) about 16 μ cal/cm^2/min.

In conclusion, one can see that though considerable data exists in the literature, no one group has done the definitive series covering heat, phosphates, oxygen, and ion movements in interrelated, internally consistent experiments. The general conclusions that can be drawn are:

1. Maintenance of excitability of all nervous tissue depends on the presence of oxygen.
2. Excitability is not affected for short periods during metabolic inhibition but eventually fails over a period of time depending upon metabolic rate (temperature), kind of nervous tissue (cell bodies or axons), and perhaps the source of nervous tissue (vertebrates vs invertebrates).

3. The action potential generating mechanism is separate and distinct from an active ion pumping mechanism. The former is dependent upon ion gradients maintained by the latter, which in turn is dependent upon metabolism.

4. The active ion pumping mechanism is in part Na^+-K^+ activated ATP'ase, and the liberated ADP may constitute the stimulus to respiration, or feedback for cellular metabolic control.

5. The energy cost of nervous activity appears to be dependent upon the surface area of the cell, the rate of ion pumping required to maintain the steady state, the electrochemical gradient against which the ions are pumped, the amount of ion movement during action potential generation, and the efficiency of operation of the ion pumping mechanism.

INTRODUCTION

PART III - EXPERIMENTAL DESIGN

The first two parts of this introduction presented physiological and biochemical evidence that neuron excitability depends upon oxidative metabolism. This oxidative metabolism in part provide the ATP that drives the sodium pump. The sodium pump maintains precise ion concentration gradients that produce the resting membrane potential. Furthermore, the generation of action potentials results in ion movements, and the sodium pump activity can be seen to increase indirectly its pumping rate in order to restore the ion distributions prior to the passage of the action potential. Most of the experimental observations in support of the above scheme have been made on isolated axon segments. Relatively little work appears on systems where synaptic transmission is a criterion of function.

The spinal cord is a tissue that is reasonably well defined physiologically. This CNS tissue contains cell bodies which have extensive dendritic aborizations and larger membrane surface areas than axons. The great difference in spinal function compared to axonal function is that synaptic transmission is required for reflex conduction. There is much literature on the effect of hypoxia on mammalian cord, but such preparations require *in situ* studies and are influenced by the systemic effects of the induced hypoxia. The isolated frog spinal cord preparation offers the simplicity of isolation from systemic influences by the organism, and capability of controlled experimental conditions not possible with *in situ* preparations. Because this tissue is isolated, experimental procedures such as temperature change and application of metabolic poisons are possible. No estimates of the energy cost of nervous activity or the relationship between temperature and hypoxic survival time have been described for central nervous system tissue. Furthermore, there exists only one short note describing the electrophysiologic consequences of hypoxia in frog spinal cord. In order to even begin molecular correlations, experiments were designed to study the behavior of reflex activity in various anatomical pathways. This thesis will present patterns of change in reflex activity observed when the isolated cord is exposed to conditions designed to upset oxidative metabolism or ion transport. These experiments were done in order to test hypotheses concerning the site of hypoxic reflex failure and possible mechanisms by which this failure could occur.

Other experiments were performed to attempt a qualitative estimation of the metabolic energy costs of this CNS preparation. If one considers the spinal cord as a metabolic black box (see Figure 24) it is apparent that there are at least two metabolic rates. The first is the metabolic rate at rest, and the second is the added metabolic rate induced by nervous activity. If a resting spinal cord is exposed to hypoxia, the synthesis of ATP might be expected to be drastically reduced, and ion gradients would not be maintained. If the energy cost of nervous activity requires a significant drain of synthesized ATP, then the failure of excitability would be expected to occur earlier if the cord was stimulated during hypoxia. If the resting metabolic rate is very high relative to the increases caused by stimulation, then changes in resting metabolic rate should affect the survival of the hypoxic cord more than changes in nervous activity. If the regions of high surface area/volume require the greatest expenditure of metabolic energy, then block of the spinal reflex should occur in the fine terminal branches of the afferent axon.

With the above hypotheses in mind, the following experimental procedures were implemented.

1. The technique of isolation of an intact, functioning frog spinal cord was refined.
2. A parameter of reflex function was established.
3. Characteristics of reflex activity to different stimuli and at different recording sites were examined.
4. The isolated cord was tested for hypoxia during the control (oxygenated) conditions.
5. The electrophysiologic characteristics of hypoxic reflex failure were examined.
6. The survival time of reflex activity at different rates of stimulation was determined.
7. The survival time of reflex activity at different temperatures was determined.
8. The anatomical site of failure of the reflex response was sought by micro-electrode techniques.
9. Evidence for the existence of a sodium-potassium ion activated ATP'ase was obtained.
10. The affect of ouabain, on active ion transport inhibitor, on reflex activity was studied.

METHODS AND MATERIALS

REFLEX ACTIVITY

The Preparation

The basis of this these studies lies in the successful isolation of a functioning frog spinal cord. Consequently, techniques for handling of frogs and for isolation of the cords are important. The animals, Rana pipiens, pipiens or grass frogs were obtained from the Powell Laboratories, Division of the Carolina Biological Supply Company, Gladstone, Oregon. These frogs, shipped from Minnesota, were specified as medium small in size (two to three inch in body length). Upon arrival, they were transferred from the shipping box to a plastic dishpan with a portion of the moss originally packed with the frogs. At this time each frog was inspected for "red leg" and other signs of disease; any that showed obvious signs of ill-health were discarded. Tap water was added to the wire-covered dishpan and this cage was kept tilted so that the water pooled to one end. The water was changed every two or three days and the moss rinsed and left in the cage. Though the frogs were usually used within two weeks of arrival, when stored in this fashion, they were viable for months.

Usually a lively frog was selected for dissection, decapitated with scissors, the skin stripped from the body, and the dorsal body wall of the frog, including the urostyle, was severed from the carcass. The body wall was pinned down in a suitable dissection chamber ventral side up, and a stream of oxygenated Ringer solution

(18° C) was directed over the ventral surface. After removing the adherent connective tissue and blood vessels from the surface of the vertebral column, a pair of cuticle nippers was employed to quickly nip away the major portions of the centra of the vertebrae. Next, remaining portions of the centra and parts of the laminae were nipped away with care to avoid damage to the underlying spinal cord. With the ventral portion of the vertebral column removed the cord is exposed. Spinal nerve X exits near the articulation of the urostyle with the sacrum. By careful dissection with iris scissors and fine tipped forceps, the ligaments attaching the nerve and dorsal root ganglion in this region may be severed. The spinal nerve X may be cut at the lumbosacral plexus, freed from connective tissue and autonomic ganglia, and lifted to aid freeing its dorsal root ganglion. Care must be taken not to pull on the nerve to prevent tearing the roots at the root entry zone of the cord. Usually both spinal nerves of level X were isolated. The cord was severed between level V and VI, the narrowest portion of the spinal cord between the cervical and lumbar enlargements. It was then lifted from the bed of the spinal canal at the transection, and the remaining roots sequentially cut to free the cord and the attached nerves of level X. The extreme caudal end of the cord was cut where it enters the urostyle and the cord transferred immediately to the experiment chamber. The oxygenated solution which had been flowing over the cord during the dissection was switched to the experiment chamber and the cord positioned for electrode placement.

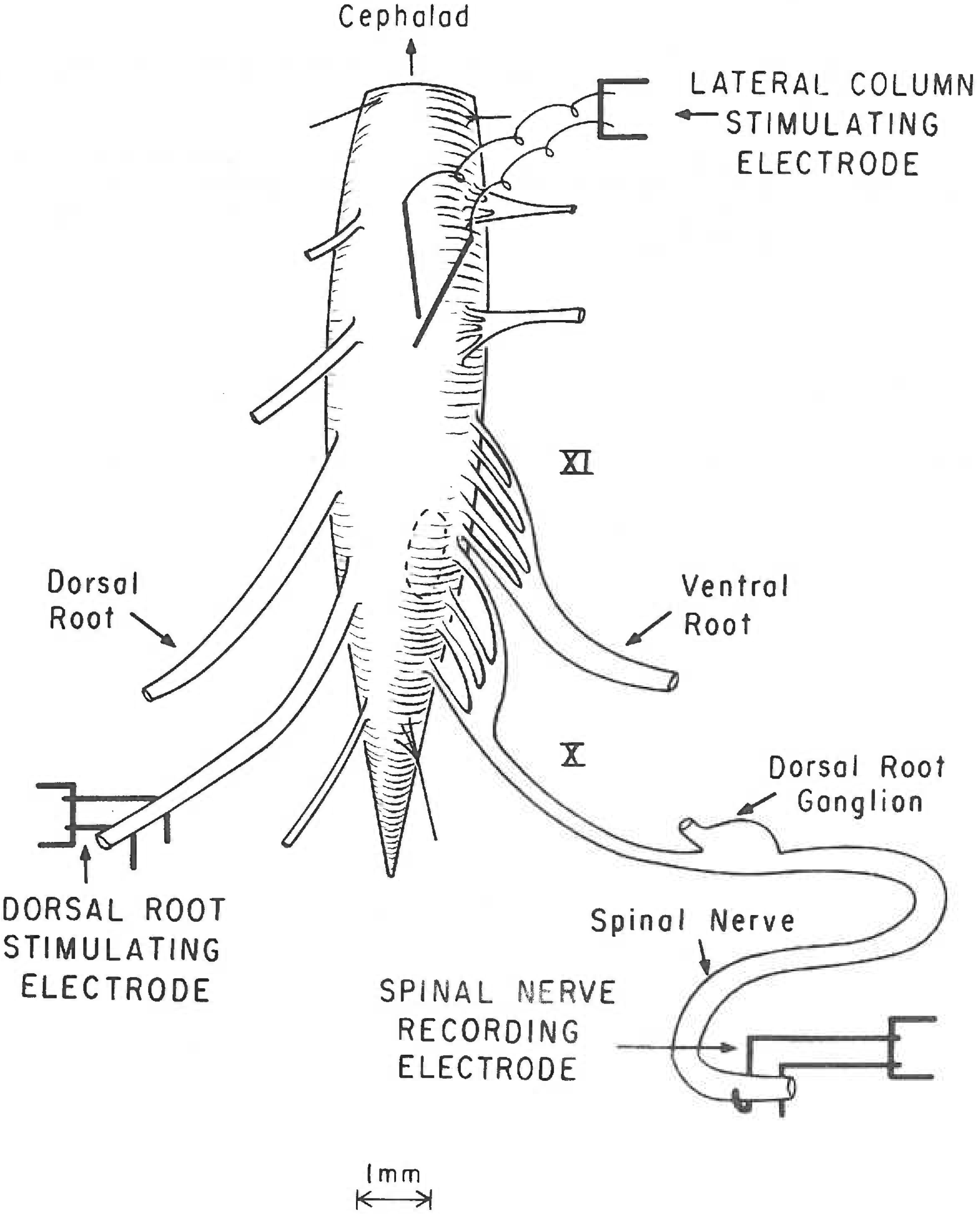
This whole dissection procedure must not take longer than 30 minutes if the cord is to be excitable. With practice the dissection could be accomplished in 7-12 minutes. Microscopic aid (7.5 X) was routinely employed, and the preparation was inspected for obvious signs of mechanical damage and morphological abnormalities. If any were found or if the dissection took longer than 20 minutes, the preparation was discarded. Typically the roots of level X are about 2/3 the diameter of those level IX, but they are longer and the dorsal root ganglion is easier to free from the intervertebral fascia. The roots of level X are about 1 cm long and the spinal nerve is about 2.5 cm long. The isolated cord weighs approximately 50 mg. All studies were carried out on level X; preliminary observations did not reveal functional differences between level X and IX. Figure 10 contains a scaled diagram of the preparation depicting its gross morphology.

Physical Environment and Experimental Conditions

The experiment chamber was 4 cm square and 2 cm deep. The trough in the chamber floor channeled the continously flowing Ringer solution past the cord. The cord was elevated from the floor by a fine wire mesh screen or nylon net to permit flow beneath as well as over the surface of the cord. A platinum ribbon served to ground the cord through the Ringer solution. Ball and socket electrode holders mounted in the chamber walls permitted stable

FIGURE 10 - RIGHT LATERAL VIEW OF THE ISOLATED FROG SPINAL CORD

This Figure shows the relative size of the roots at the different spinal levels (Roman numerals). The dotted area encircles the region through which the micro-electrode is inserted for focal potential recording.


Cephalad
LATERAL COLUMN
STIMULATING
ELECTRODE
XI
Dorsal
Root
Ventral
Root
X
Dorsal Root
Ganglion
DORSAL ROOT
STIMULATING
ELECTRODE
Spinal Nerve
SPINAL NERVE
RECORDING
ELECTRODE
1mm

placement of the roots and nerves on electrodes isolated from ground by a piece of Parafilm. The chamber and lid were shielded with grounded aluminum foil.

Once the dorsal root and spinal nerve were positioned on the electrodes, their exposed surfaces were covered with a mineral oil petroleum jelly mixture to prevent drying. The portion of the cord to be stimulated by the lateral column electrodes was raised just above fluid level with a tiny cotton wad; a little of the dura was removed. Bipolar stimulating electrode tips were inserted subpially along the mid-lateral longitudinal axis of the cord. This last step was found to be critical for effective production of the lateral column reflex. When micro-electrode recording was done, the dura was removed from the left lateral surface of the cord. The pia was carefully split on the mid-lateral line with fine tipped forceps. A glass micro-electrode was mounted in a micro-manipulator, lowered into the chamber through a hole cut in the lid, and inserted into the cord under microscopic observation.

Two Ringer solutions of identical ionic composition were prepared (see Appendix). One solution was saturated with 95% O_2-5% CO_2 and the other with 95% N_2 - 5% CO_2 for at least 1.5 hours before an experiment. The desired gas mixtures were passed continuously through the siphon bottles using a gas dispersion tube to aid in complete saturation before and during the experiment.

Fluid from the bottles passed through a plasma dropping tube calibrated to measure flow (3-6 ml/min) and controlled by a screw clamp. The Ringer solutions passed through several coils of Tygon tubing immersed in a cooling bath, and were delivered to the chamber via a three-way stopcock. This stopcock could be turned to admit either oxygenated or nitrogenated Ringer solutions. During hypoxia, 95% nitrogen-5% CO_2 gas was admitted to the chamber to deoxygenate the atmosphere. The temperature of the system was monitored by a metered thermistor bridge and a Grass polygraph. Low temperature calibration was checked at six month intervals and found to require only a minor adjustment of the bridge. Room temperature recorded from a thermometer was used to check the high temperature calibration. Temperature was measured with a precision of 0.5 degrees Centigrade and an accuracy of better than 0.1 degree Centigrade.

Subambient temperature was produced in the cooling bath by a metal cold finger. Usually it was sufficient to circulate cold tap water through this cold finger to produce bath temperatures in the desired range (15-19° C). However, during the summer months, or when very low temperatures were needed, it was necessary to circulate cold water from a second bath. This second bath was cooled either by ice, or by a thermoelectric cold finger (C. Thomas and Co.). Fine control over the chamber temperature (± 0.5° C) was attained by varying the combination of water flow through the cold finger and Ringer flow through the cooling bath and chamber. Flow was readjusted whenever the temperature drift was seen to approach 2° C. Ringer flow was never allowed to be less than 3 cc/minute and was usually around 5 cc/minute. The mean temperature during the control (oxygenated) and experimental periods (unoxygenated) was not permitted to differ by more than 2° C.

Instrumentation for Measuring Reflex Activity

Electrodes for stimulating the dorsal root and recording from the ventral root or spinal nerve were made with 0.01 inch platinum wire. Insulated leads to a phone plug connector were soldered to the wire and affixed to a suitable glass tube for insertion into the electrode holders of the experiment chamber. The lateral column stimulating electrodes were fashioned from 000 insect pins insulated with Insulex except at the tips. Again, appropriate leads and connectors were fastened to the electrode.

Micro-electrodes were drawn from glass capillary tubes on a micro-electrode puller. The pipettes were filled with alcohol by vacuum boiling. The alcohol was subsequently replaced with two molar NaCl by diffusion. (Sometimes the electrodes had a bubble visible in the tapering shank of the pipette that could be safely dislodged by flicking a finger against the electrode). Electrodes with resistances between two and ten megohms were used.

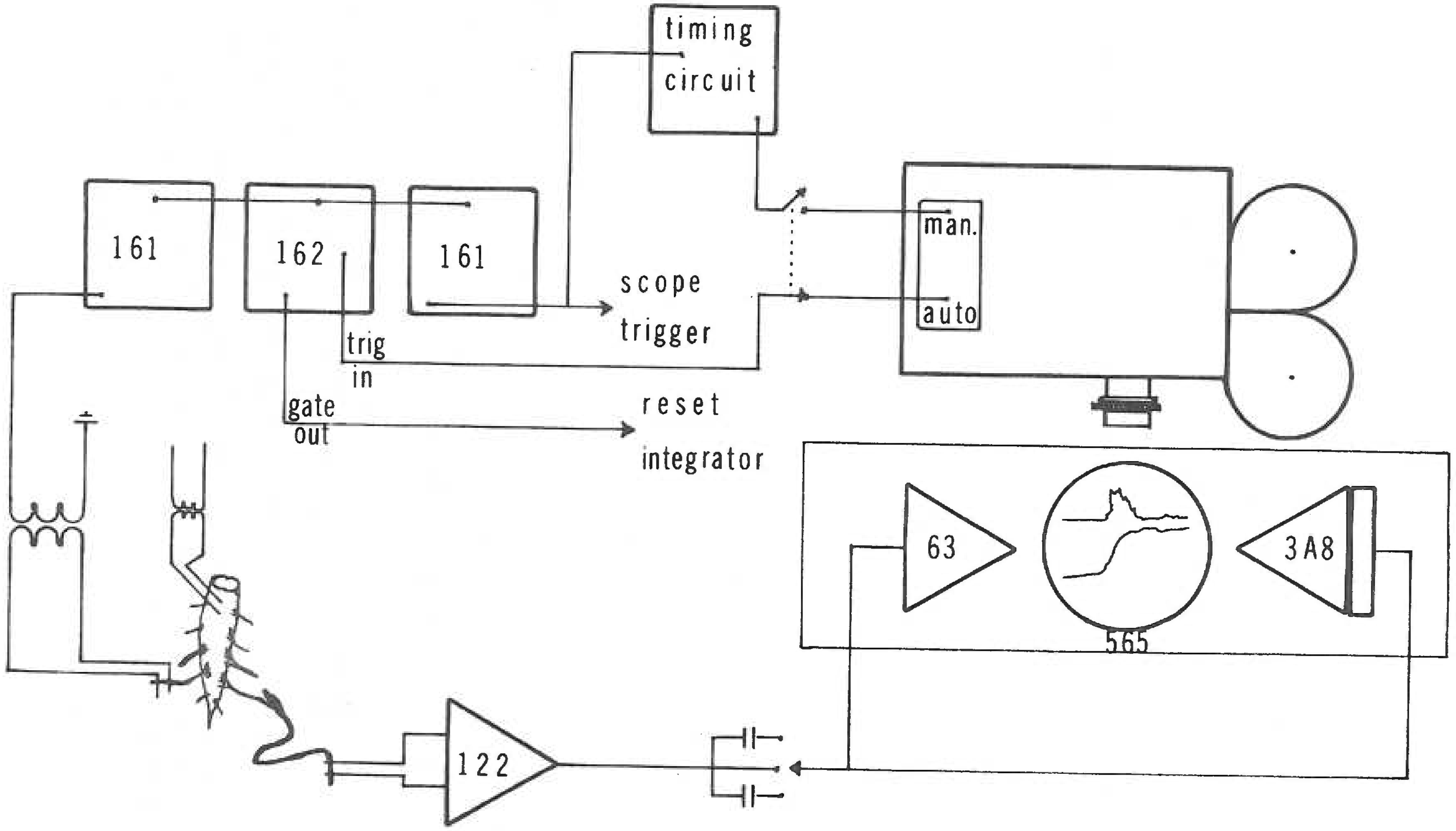
The instrumentation used to measure reflex activity in the isolated spinal cord is presented diagrammatically in Fig. 11. The stimulus pulse is obtained from a Tektronix Type 161 pulse generator and sent to the dorsal root or lateral column through an isolation transformer.

FIGURE 11 - SCHEMATIC OF THE INSTRUMENTATION

This arrangement was used in most experiments. When microelectrode recording was performed, another stimulator and an electrometer were added to the above system. In addition, a dual beam plug-in unit (Tek Type 3A3) replaced the Tektronix Type 63 vertical plug-in amplifier.

Various trigger circuits were designed and built for stimulus selection and timing. Schematics for these circuits can be found in the Appendix.

Instrument numbers refer to Tektronix Type numbers. The camera is a Grass Inst. Co., model C4.


timing
circuit
161
162
161
scope
trigger
trig
in
gate
out
reset
integrator
man.
auto
63
3A8
565
122

Reflex motor neuron discharge in the nerve or root was amplified by a Tektronix Type 122 differential preamplifier with a gain of 5,000 and a Type 63 vertical plug-in amplifier. Display of the reflex discharge was obtained from a Tektronix 565 dual beam oscilloscope. The amplified nervous activity from the Type 122 was also channeled to a Type 3A8 operational amplifier plug-in unit. The operational amplifier was used to integrate the reflex discharge, and this was displayed on the second channel of the dual beam oscilloscope.

It was necessary to be able to record with reasonable accuracy reflex responses having peak amplitudes as low as 100 mv. The high gain required for the proper integration of these low level responses made it necessary to take unusual precautions to avoid electrical variations of non-biological origin. Therefore, the system was modified in the following way. To prevent mechanically induced noise, the nerve recording electrode leads were soldered directly to the amplifier input cable. The input connector of the preamplifier was changed to accommodate a Bendix four pole screw type connector. The band pass of the preamplifier was reduced to values that did not significantly affect the wave form or amplitude of the reflex discharge signals. The band pass was normally $0.8h_z$-1 Kh_z. A fine control potentiometer for the output D.C. level was added to the preamplifier. The output of the preamplifier was connected to a three pole shielded switch that would couple the Type 63 and 3A8 amplifiers directly (D.C.) or by capacitors

(1.0 and 0.2 μf). These capacitors prevented D.C. drift in the output stage of the preamplifier from interfering with the integration of the biological signal without affecting the frequency response. Other procedures such as common grounding, proper power supply location, elimination of room lighting, and electrode shielding helped achieve a noise level of approximately 10 microvolts peak to peak over the band pass of the instrumentation.

When micro-electrode recording was employed, a Medister A-34 electrometer was used in conjunction with a Tektronix Type 3A3 dual trace amplifier.

Permanent records were made by photographing the face of the oscilloscope with a Grass C4 oscillographic camera. In experiments where the stimulus interval was 40 seconds, the camera was also used as a pacemaker to synchronize stimulation and display of the response for photography. The camera was set to take pictures at 40 second intervals. When the shutter opened, a spike was generated by the camera which triggered a Tektronix Type 162 wave form generator that in turn triggered the oscilloscope sweep and the stimulus pulse. A gate circuit on the Type 162 was used to reset the 3A8 integrator by closing a relay to short out the feedback capacitor of the operational amplifier. This relay opened 50 msec. prior to the stimulus pulse and closed 4 seconds later. Each picture taking event was recorded on the timebase of a Grass polygraph. Because the shutter of the camera opened for 1/2 second, the actual interval between stimuli is 40.5 seconds. When it was desired to stimulate at 4 second intervals, a special counting circuit was designed to trigger the camera automatically (see Appendix). This circuit allowed the camera to photograph four consecutive responses out of a set of sixteen. Another special gating circuit for use during micro-electrode experiments was constructed to select in sequence three different stimulators. During those experiments, stimuli would occur at 40 second intervals. The stimulus sequence was lateral column, dorsal root, lateral column, antidromic, etc. Thus, there was an 80 second interval between successive lateral column stimuli and a 160 second interval between successive dorsal root or antidromic stimuli.

Stimulus characteristics: All stimuli, unless so stated, were supramaximal rectangular pulses which did not exceed 6 v out of the isolation transformer. The pulse widths for lateral column stimuli varied from 0.01-0.15 msec. Pulse width for dorsal root was usually 0.10 msec. and for antidromic, 0.01-0.05 msec.

Recording polarity: In all traces an upward deflection indicates negativity relative to the killed end of the root for bipolar recording or nerve bathing solution for micro-electrode recording.

Thus, the instrumentation provided for automatic stimulation of the isolated cord, displayed the response, and photographed the results at precise intervals. The Grass polygraph continuously displayed the temperature and temporally monitored the events. The photographic record was examined in a film reader at 3.5 x the original magnitude and the amplitude of the discharge and integrated discharge was measured. In the case of four second interval stimulation, the average of three responses in each set of 16 was taken for a single data point. All measurements were made with a precision of one mm. Reproducibility was typically ± 1 mm, but in some particularly noisy records the reproducibility was ± 3 mm. The integral measures of the enlarged records ranged between 100-200 mm during the control period. Amplitude measurements of the afferent signal in focal potential records were between 10-15 mm. Since these signals usually were reduced during the experimental period, the accuracy of measurement declined, especially when the signal to noise ratio approached unity. Consequently, the measure called survival time in this thesis is based upon the length of time that the response took to fail to only 50% of the control level. Amplitude measurements of the integration trace are subject to the same limitations. By recording the gain and integration rate of the 3A8 plug-in, an estimation of the total amount of reflex discharge can be made (volt-sec.).

OXYGEN TENSION MEASUREMENTS IN SOLUTION

A polarographic technique was employed for three different purposes to assay the amount of dissolved oxygen in solution. The first purpose was to determine the amount of time it took to convert from the oxygenated control to the nitrogenated experimental condition so that the amount of onset of chamber hypoxia could be accurately estimated. A bare platinum wire polarizing electrode was positioned in the trough of the experiment chamber near a second non-polarizing Ag-AgCl electrode. A Systems Research Corp. power supply, a RCA ultramicro ammeter, and a Grass polygraph were connected as in Fig. 12A. The potential measured was proportional to the oxygen tension of the Ringer solution flowing through the trough. With no spinal cord present but otherwise normal experimental conditions, the PO_2 fell to a minimum value (6%) within 10 seconds as nitrogenated Ringer was admitted to the chamber. It was assumed, therefore, that the onset of environmental hypoxia began less than one minute after the inflow of nitrogenated Ringer solution.

The second use for oxygen tension measurements was to determine the level of oxygenation inside the spinal cord under the usual control conditions. In this case a standard Beckman Micro Oxygen Electrode was used to measure the PO_2 in the central core of the spinal cord.

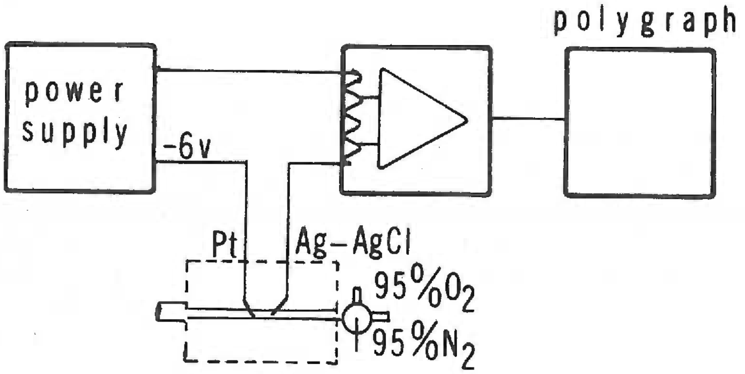
The last oxygen tension study was undertaken to measure the oxygen consumption of spinal cord tissue brei. The brei was prepared from four spinal cords (180 mg) by three firm twists in a ground glass Potter-Elevhjem homogenizer. It was added to a specially designed water-jacketed cuvette containing a standard Beckman Macro Oxygen Electrode (courtesy of Dr. H. S. Mason). (See Fig. 12B). The value of PO_2 in the cuvette was continuously monitored by the electrode system and displayed on a Sargent servorecorder. Under these conditions, the effects of glucose,

FIGURE 12 - DISSOLVED OXYGEN MEASUREMENTS

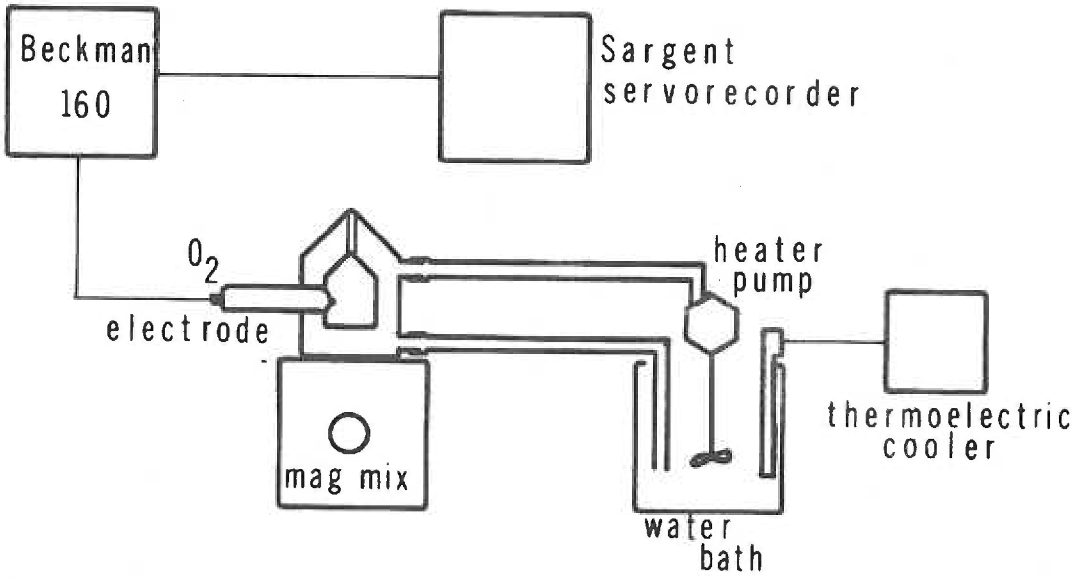
Part A: A schematic of the arrangement to measure the latency of environmental hypoxia after the start of the nitrogenated Ringer inflow. The stopcock could be turned to admit either oxygenated or nitrogenated Ringer.

Part B: Schematic for the oxygen tension measurements of spinal cord brei. The brei is pipetted into the chamber, magnetically stirred, and oxygen tension monitored by a Beckman Macro Electrode and 160 Gas Analyzer. Slope measurements were made from the records generated by the servorecorder. Temperature of the circulated water was thermostatically controlled.

A


polygraph
power supply
-6v
Pt
Ag–AgCl
95%O_2
95%N_2

B


Beckman 160
Sargent servorecorder
O_2 electrode
heater pump
thermoelectric cooler
mag mix
water bath

adenosine diphosphate (ADP), and ouabain on the oxygen consumption of the spinal cord brei were observed.

Calibrations

A Medistor low voltage calibrator was used to check the stability of the amplifier and display system. A 500 μv, 1 Kh_z signal from this device was fed into the input of the Type 122 as an attempt to simulate the recording condition during the experiments. The Type 122 had a gain of 5,000, and no measurable drift in the gain of the 122 or vertical plug-in amplifier could be detected over a period of several hours. Initially, it was routine before the start of each experiment to check the common mode, rejection, the output level, and voltage calibrations from the calibrate output on the Type 565 oscilloscope. However, these parameters required so little adjustment that they were only checked once during a week of experiments. When the Medistor 341 electrometer was used, internal calibration devices allowed gain and electrode resistance checks during the experiment.

The time base of the Type 565 was calibrated by a Type 181 time mark generator which served as a tertiary standard.

The linearity of the integrator was checked in a fashion designed to simulate the conditions during the experiment. A Tektronix Type 114 pulse generator was used as a voltage source. A rectangular pulse of variable width and amplitude was fed into a 50 ohm terminated attenuator on the input of the Type 122. Both the pulse from the input of the integrator and its integral from the output of the integrator were displayed. The integrator output was a linear function of changes in pulse width (1-40 msec.) and changes in amplitude (0-2 mv).

Before any piece of electronic equipment was incorporated into the instrumentation, it was thoroughly checked out and internally calibrated according to the manufacturers specifications.

Identification of Experimental Groups

Two principal experimental treatments were performed on frog spinal cord isolated for study of their reflex activity. There were 52 cords made hypoxic under various experimental conditions, and eight others were treated with ouabain. Of the cords made hypoxic, there were 11 preparations where observations of dorsal root and lateral column induced reflex activity were made at the same time. The data from these experiments, then, are paired observations. However, of these 11 experiments, 6 were prepared, in addition, for micro-electrode study. The pattern of hypoxic reflex failure in these two subgroups of paired observations was significantly different. Therefore, those experiments where paired observations were made, but whose cords were not prepared for micro-electrode penetration, are identified as Group I (paired observations). Those experiments where paired observations during hypoxia were performed with micro-electrode technique are identified as Group II (paired observations). Other major experimental subgroups include spinal cords whose dorsal roots were stimulated at 40 second intervals and cords stimulated at 4 second intervals. The cords responding at 40 second intervals can also be divided into three subgroups on the basis of the environmental temperature of the experiment. These temperature subgroups are: high, (20-25° C), medium (17.5-19.5° C), and low (9-14.5° C). The following outline may assist the reader in differentiation of the groups, and preparing the reader for comparisons made between subgroups in the Results section.

I. Hypoxia experiments
    A. Dorsal root induced reflex activity
        1. 40 second intervals
            a. High temperature
            b. Medium temperature
            c. Low temperature
        2. 4 second intervals (medium temperature)
    B. Paired observation of dorsal root and lateral column activity
        1. Group I
        2. Group II - prepared for micro-electrode recording
    C. Dorsal root induced activity recorded from the ventral root
II. Ouabain experiments - prepared for micro-electrode recording

RESULTS

ELECTROPHYSIOLOGIC CHARACTERISTICS OF REFLEX ACTIVITY IN FROG SPINAL CORD

Patterns of Reflex Activity

When the dorsal root of the isolated frog spinal cord is stimulated, a polysynaptic reflex discharge of the motor neurons can be detected from the ventral root or spinal nerve. Typical responses of this kind are displayed in Fig. 13, as the upper traces of A and B. When lateral column fibers just beneath the midlateral surface of the preparation are stimulated, a much different pattern of motor neuron discharge is seen from the spinal nerve. This response is highly synchronous and occurs with a much shorter latency (Fig. 13C upper trace). Previous studies (146,147,143) have shown that the dorsal root induced reflex is purely polysynaptic whereas the lateral column induced reflex is largely monosynaptic. Small amounts of polysynaptic activity following the large monosynaptic discharge can be observed in the record of Fig. 13C. If the reflex discharge in the spinal nerve is electronically integrated, records similar to those in the lower traces of Fig. 13 are observed. The amplitude of this lower trace at any point in time is directly proportional to the accumulated area beneath the discharge in curve in each upper trace. The parameter chosen as a measure of the magnitude of reflex discharge in motor neuron axons is the amplitude of the integral. For the monosynaptic reflex, the integral amplitude is measured at the moment the monosynaptic discharge gives way to polysynaptic activity.

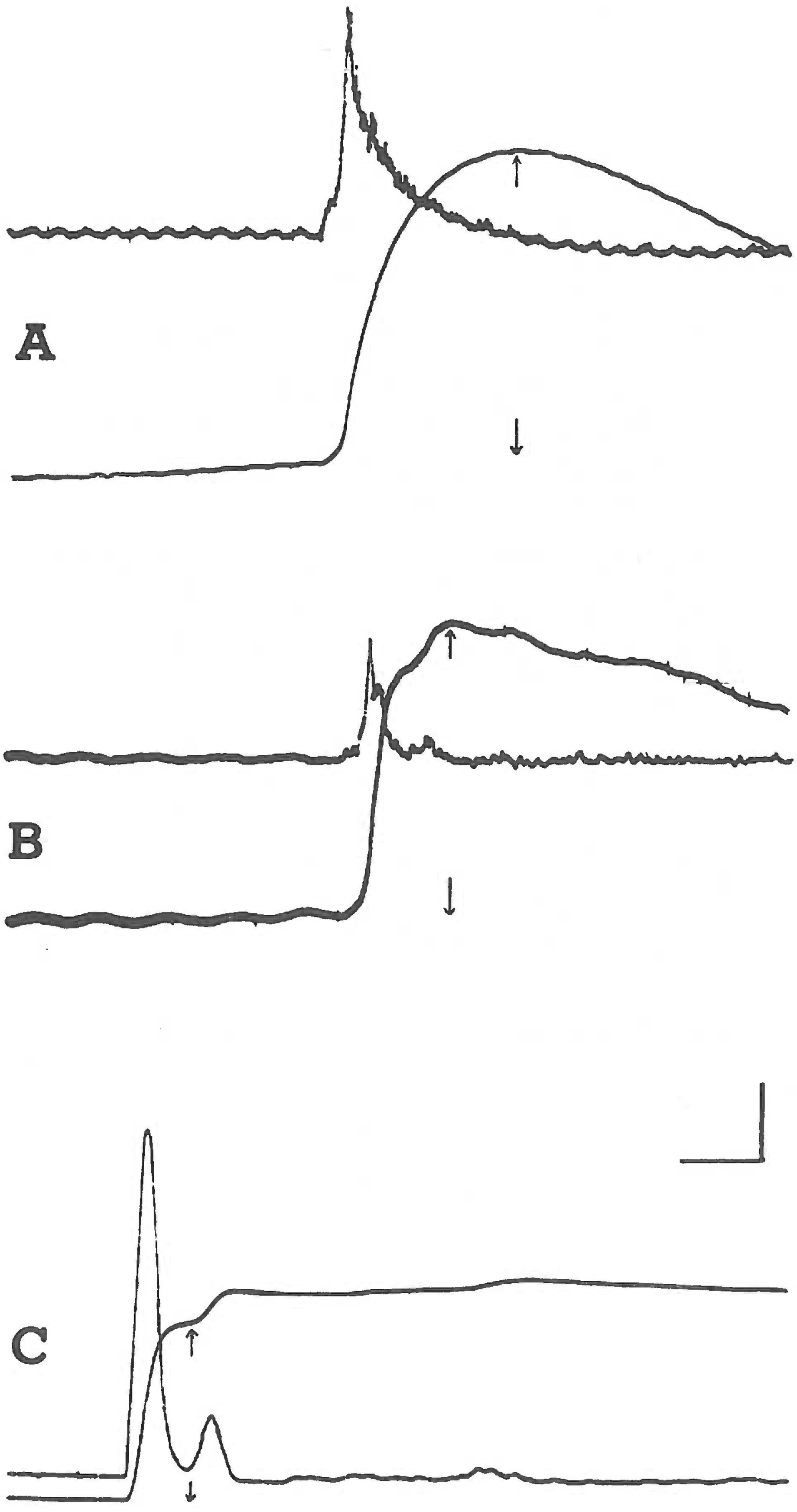
FIGURE 13. Examples of reflex potentials and their integrals in the isolated frog spinal cord.

Part A: Dorsal root induced reflex activity in the ventral root.
Upper trace - ventral root potential
Lower trace - integral of ventral root potential
Calibration mark
Amplitude - 100 µv, 10 volt-msec.
Duration - 50 msec.

Part B: Dorsal root induced relfex activity in the spinal nerve.
Upper trace - spinal nerve potential
Lower trace - integral of spinal nerve potential
Calibration mark
Amplitude - 100 µv, 1 volt-msec.
Duration - 20 msec.

Part C: Lateral column induced reflex activity in the spinal nerve.
Upper trace - spinal nerve potential
Lower trace - integral of spinal nerve potential
Calibration mark
Amplitude - 400 µv, 2 volt-msec.
Duration - 5 msec.

Note: The integral amplitude is measured between the arrows.



A
B
C

It is important to consider the differences between the responses to dorsal root stimulation represented by Fig. 13, A and B. One can note that the time course and amplitude of the response detected in the ventral root is much longer and larger than when recorded distally on the spinal nerve. Previous reports indicate that a large potential change can be detected in the ventral root (A) in response to dorsal root stimulation even though discharge is blocked by pentabarbiturate (149). This potential change is due to summated excitatory postsynaptic potentials (EPSP) electrotonically conducted out the ventral roots. As shown in the introduction, electrotonic potentials decrease in amplitude as the measurements are made at greater distances from the source. The spinal nerve record was obtained at about 3 cm from the cord in contrast to 1 cm for the ventral root recording. The added distance for spinal nerve recording decreases the amount of electrotonic potential in the record and provides a better representation of the pattern of discharge propagated in the motor axons since it is uncontaminated by electrotonically conducted EPSP's and propagated axon potentials. One of the key elements of data in this study is an estimate of the amount of discharge produced by excitation of reflex pathways under various conditions. To facilitate the evaluation of changes, the amount of electrical activity induced by two varieties of excitation has been measured under controlled conditions.

Magnitude of Reflex Activity

Table III is concerned with dorsal root induced reflexes only. There was no difference in the magnitude of discharge when recorded from the spinal nerve at stimulus intervals of 4 or 40 seconds. However, the reflex magnitude was observed to decline when the interval between stimulus was less than 2 seconds. At similar temperatures, median values of discharge recorded from the ventral root were significantly larger ($p < 0.001$) than median values of discharges recorded from the spinal nerve. This suggests that the electrotonically conducted potentials account for about 80% of the total potential change observed. The data in Table IV indicates that the magnitude of reflex discharge was significantly larger ($p < 0.001$) at low temperatures than at moderate. There is no significant difference between observations at low (9.5-14° C) and high temperatures (20-25° C). (Unless otherwise stated, the two tailed rejection region for the null hypothesis is $p \leq 0.05$). In general the standard deviations were surprisingly small considering the technical difficulty in making absolute measurements from different preparations.

In Table V, the magnitude of reflex activity induced by dorsal root stimulation is listed with magnitudes from lateral column stimulation. These observations are from control periods of 19 experiments in which both types of reflexes were produced in the same preparation. Therefore, these values constitute paired observations. Though the mean values do not appear significantly

RESULTS

TABLE III

MAGNITUDE IN VOLT-SEC. 10^{-2} OF DORSAL ROOT INDUCED REFLEX DISCHARGE (15-20° C)

Experimental Conditions	Range	Median	Mean	S.D.	N
Stimulus Interval 40 sec.					
Ventral root	2.76-3.83	2.85	3.15	0.59	3
Spinal nerve group I	0.31-0.76	0.50	0.49	0.14	9
Spinal nerve group II	0.34-2.62	0.55	1.00	0.76	11
Stimulus Interval 4 sec.					
Spinal nerve	0.46-4.27	0.60	1.24	1.37	9

TABLE IV

MAGNITUDE IN VOLT-SEC. 10^{-2} OF DORSAL ROOT INDUCED DISCHARGE IN THE SPINAL NERVE AT VARIOUS TEMPERATURES

Temperature Range °C	Range	Median	Mean	S.D.	N
9.5-14.0	0.56-2.2	1.35	1.48	0.69	5
17.5-19.5	0.31-0.76	0.50	0.49	0.14	9
21.0-25.5	0.23-2.4	0.40	0.76	0.79	5

TABLE V

MAGNITUDE (IN VOLT-SEC. 10^{-2}) OF SPINAL NERVE DISCHARGES INDUCED BY DORSAL ROOT AND LATERAL COLUMN STIMULATION (N=19 PAIRED OBSERVATIONS; 15-20° C)

Stimulus	Range	Median	Mean	S.D.
Dorsal root	0.34-2.62	0.67	0.95	0.67
Lateral column	0.32-2.57	0.77	0.99	0.52

different, there were only five cases in which the dorsal root induced reflex achieved a greater magnitude than the lateral column. The probability that the reflex magnitudes from both stimuli will be the same in any experiment can be computed from the binomial (sign) test, where p=0.32 (one tailed). Although the difference was not great the dorsal root induced response was frequently smaller than the response to lateral column stimulation. The relative magnitudes remained the same throughout the course of any one experiment. Further implications derived from observations of reflex magnitude will be considered in the discussion section of this thesis.

The amount of electrical potential recorded from platinum electrodes placed on the ventral root or spinal nerve depends upon several factors. The inter-electrode distance, the inter-electrode resistance, and the amount of inactive tissue between active axons and the active electrode are physical factors which affect the magnitude of recorded potentials. Attempts were made to reduce the variability of these factors by maintaining the same inter-electrode distance, using the same spinal level, rejecting preparations with unusual morphology, and affixing the preparation in the chamber in a similar manner with each experiment. The variability in measurements observed among preparations in any one group probably results more from variations in these physical factors than from biological variation.

Durability of Reflex Activity

Previous studies indicate that this preparation, when handled in the manner already described, will generate reflex activity for "four to five hours without signs of deterioration" (147). Responses to dorsal root stimulation have been observed for as long as 19 hours (150). The latter study cites experiments on isolated frog spinal cord that were carried out over a period of two days [(150), page 71]. Control experiments resulted in complete assurance that the preparations used in this study were as durable as had been reported previously. Furthermore, control periods prior to experimental manipulations were of sufficient duration to assure that the preparations studied were viable and were not showing significant signs of deterioration by reduction in the magnitude of reflex activity.

It might occur to some that the spinal cord was hypoxic during the control period in spite of the good evidence for durability of the preparation cited above. Attempts were made to measure the PO_2 in the central core of the isolated cord while bathed in oxygenated Ringer. Serious technical problems were encountered. A commercial Clarke type micro oxygen electrode was used in two preparations. In both preparations the PO_2 readings varied abruptly and erratically between zero or about 100 mm Hg. The PO_2 of the bath was measured at 600 mm Hg. The zero readings were observed as the electrode was pushed into the canal. Abrupt changes between high and low readings could be induced by mechanical manipulations of the electrode. Similar observations were made when the electrode was forced through a thin piece of parafilm. It was concluded that the membrane of the electrode made the recordings mechanically sensitive. To test this possibility, the electrode was used without the membrane in place in full knowledge of the fact that the electrode was not designed to be used in this fashion. Errors introduced due to the higher than normal resistance in the current pathway, or the formation of a protein scum on the electrode, would lead to an underestimation of PO_2. In spite of these possibilities of error, the membraneless electrode was calibrated in Ringer solution at the cord temperature and quickly forced into the central canal to obtain a brief reading. It was then removed, recalibrated, and another reading was taken. Values varying between 13 and 30 mm Hg were obtained at a time when the external PO2 was 600 mm Hg. Since it was obvious that a special micro oxygen electrode would have to be designed, constructed, and calibrated to provide precise values, further attempts at direct measurement were abandoned. These experiments suggest, however, that there is a detectable level of PO_2 even in the inner most regions of the spinal cord.

PATTERNS OF REFLEX FAILURE DURING HYPOXIA

Polysynaptic Reflexes

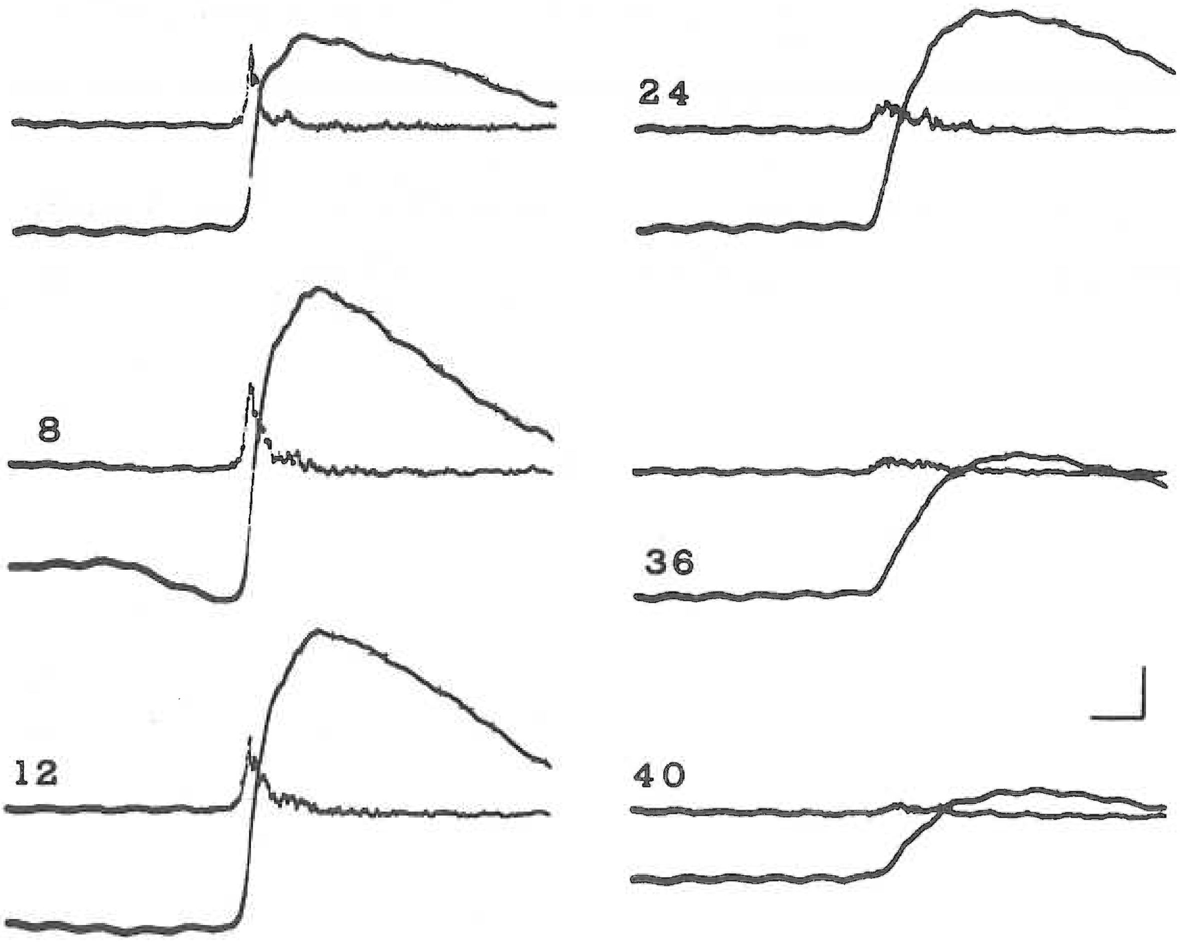
Fig. 14 illustrates selected records from a typical experiment in this series. Following a period of normal, stable responses recorded from the spinal nerve, the onset of hypoxia was associated with changes in the magnitude of the responses to dorsal root

FIGURE 14. Records of dorsal root induced reflex activity during hypoxia.

Upper left hand record - control period subsequent records numbered in minutes after the start of hypoxia.

In each record:

Upper trace - spinal nerve potential
Lower trace - integral of spinal nerve potential
Calibration mark
  Amplitude - 100 µv, 1 volt-msec.
  Duration - 20 msec.



24
8
36
12
40

stimulation. Inspection of such records failed to reveal changes in the latency of the reflex discharge. In addition, the duration of the reflex discharge did not undergo marked alteration. The most obvious changes in the discharge typically consisted in an alteration of the earliest elements of the complex discharge pattern. The first few minutes of hypoxia were usually characterized by an augmentation of activity. During the succeeding minutes activity diminished gradually, reaching a very low level in 30-40 minutes. A more generally applied method of describing these changes is illustrated in Figure 15. One can see that the magnitude of the integral was subject to less fluctuation than the peak voltage attained during the discharge recorded from the spinal nerve. This experiment showed an initial augmentation of the discharge magnitude occurring about 10 minutes after the onset of hypoxia. Following this augmentation there was a gradual non-linear decline of reflex activity. The survival time, or time from onset of hypoxia to 50% decay in response, was obviously different, when peak amplitude and discharge integral are compared. This is understandable when it is recalled that the instantaneous voltage reflects the number of elements simultaneously active. Thus, peak voltage changes reflect the degree of synchronization; this may fluctuate widely from response to response. The integral of discharge, on ther other hand, is a function of the sum of all individual responses over this time of measurement and, in my view, constitutes a more satisfactory measure of the total discharge. This relation between amplitude and

FIGURE 15. Time Course of Polysynaptic Reflex Failure

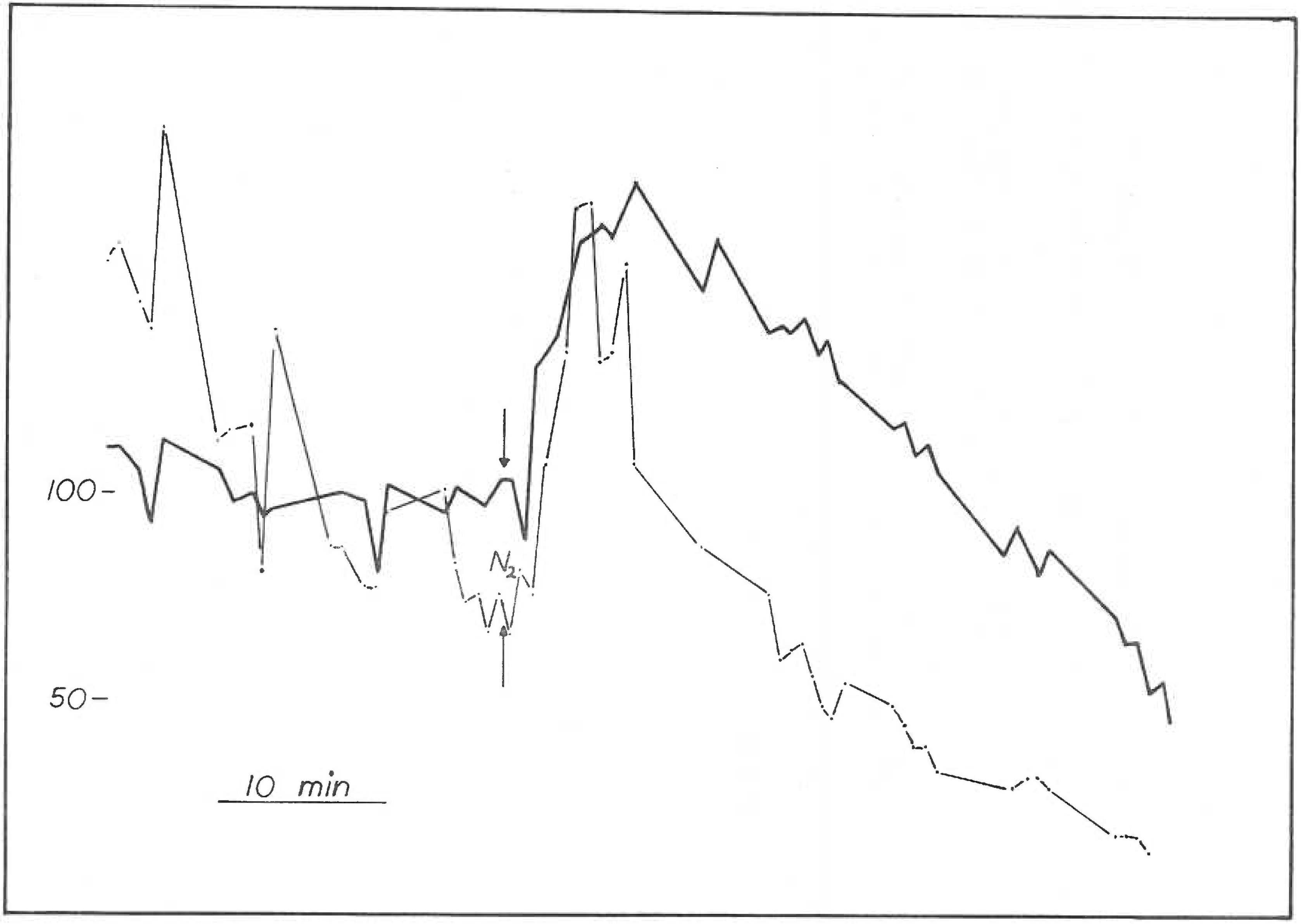
The heavy solid line is the integral of the spinal nerve potential (% of control) plotted vs time.

The light dashed line is the peak amplitude (arbitrary units) of the spinal nerve potential plotted vs time for comparison with its integral.

These potentials were reflexly induced by stimulation of the homolateral dorsal root.

(The data was obtained from the same experiment illustrated in Figure 14).

Onset of the hypoxic period marked by arrows.


100-
50-
N2
10 min

and integral is clearly evident in the data illustrated in Fig. 16. The response (inset) recorded at 8 minutes showed a marked increase in peak voltage and a reduction in duration. These two changes resulted in only a relatively small change in the magnitude of the integral. Fig. 16 is a graph of the results from three experiments in which dorsal root induced reflex was recorded from the ventral root instead of the spinal nerve as was the case in Fig. 15. In the experiments whose integrals are shown in Fig. 16, the peak amplitude in all cases showed an initial augmentation, shortly after the onset of hypoxia, but the integrals showed an initial depression. This result is in sharp contrast to experiments where measurements were made from the spinal nerve (see Fig. 15). Consequently, it was decided to confine recordings of reflex activity to the spinal nerve because; (1) the center of interest in this study is magnitude of discharge and (2) the electrotonically conducted potential changes cannot be eliminated from ventral root recordings.

Monosynaptic Reflex

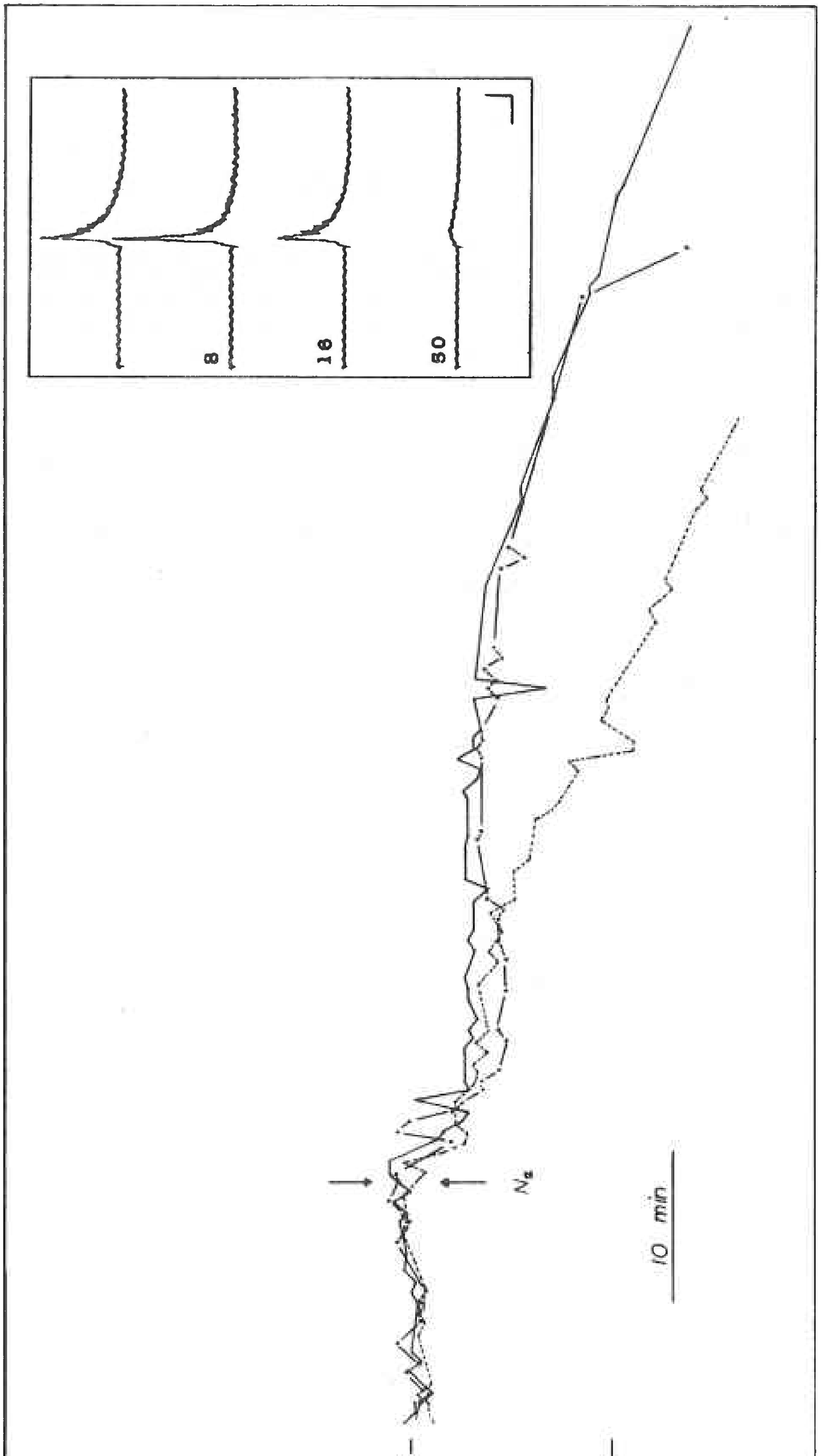
In a number of experiments the effects of hypoxia on the lateral column induced (monosynaptic) reflex were examined. These experiments were technically much more difficult than were the dorsal root (polysynaptic) studies. Slight mechanical disturbances could upset the stimulus conditions sufficiently to destroy the value of the experiment. It appeared that minor uncontrolled variations in the position of the stimulating electrode in the lateral column were

FIGURE 16. Reflex Activity in the Ventral Root

The integrals of the ventral root potentials (% of control) are plotted vs time for three separate experiments. Ventral root potentials were produced reflexly by supramaximal stimulation of the homolateral dorsal root.

Inset: Four records from one of the three experiments plotted below. These records have had the integral deleted to show more clearly the pattern of activity and the changes in this pattern during hypoxia. Upper most record is from the control period, and the numbers indicate the minutes after onset of hypoxia when the record was made.

Calibration: 500 μv, 50 msec.


100
50
N₂
10 min
8
16
50

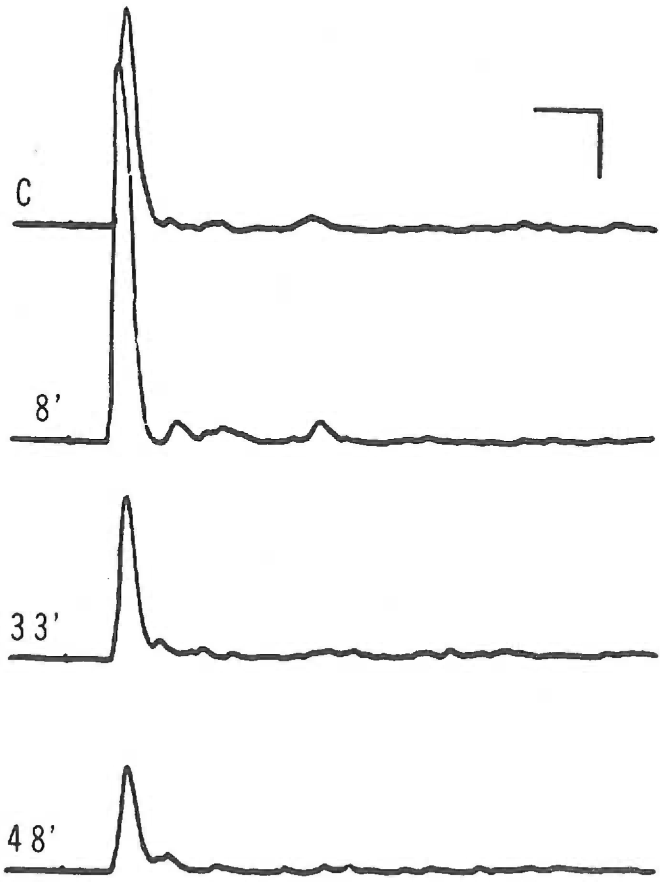
associated with major changes in the amplitude and time course of the discharge. The experiments which were discarded were characterized by abrupt changes in the pattern of discharge which could only be explained on this basis. Nevertheless, 19 experiments were successful in that the responses during the control period were stable and alterations during hypoxia were not marked by sudden and reversible changes in discharge pattern or magnitude. Four records from one such experiment are shown in Fig. 17. The portion of these records produced by monosynaptically induced discharge is the initial larger negative potential and is followed by a series of lesser potential fluctuations which represent motor neuron discharge induced via polysynaptic pathways. The augmentation of discharge which occurred during the first 10 minutes of hypoxia was seen in all Group I experiments but it was never as great as the augmentation of the dorsal root induced (polysynaptic) reflex in the same preparation. The median values for the maximal levels of reflex activity achieved during hypoxia were 130% and 150% of control in monosynaptic and polysynaptic observations respectively. Both types of reflexes reached their peak augmentation at about the same time (10 minutes). The failure of the reflex was gradual and without any marked change in the duration of the discharge. It would thus appear that reflex failure, examined in terms of pattern and magnitude of the evoked discharge, occurs in the same fashion in both simple (monosynaptic) and more complex (polysynaptic) reflex

FIGURE 17. Monosynaptic Reflex Activity During Hypoxia

Four records from one experiment. The integrals have been deleted in order to demonstrate the details of the discharge in the spinal nerve.

Top trace - control record.
Lower traces - numbers indicate minutes after onset of hypoxia.

Calibration: 400 μv, 5 msec.


C
8'
33'
48'

pathways, i.e., by a gradual reduction in magnitude without remarkable changes in time course. Figure 18 is a composite of the integral measurements of monosynaptic reflex discharge taken during hypoxia in five separate preparations not prepared for micro-electrode studies (Group I).

Polysynaptic vs Monosynaptic Reflex Survival Times

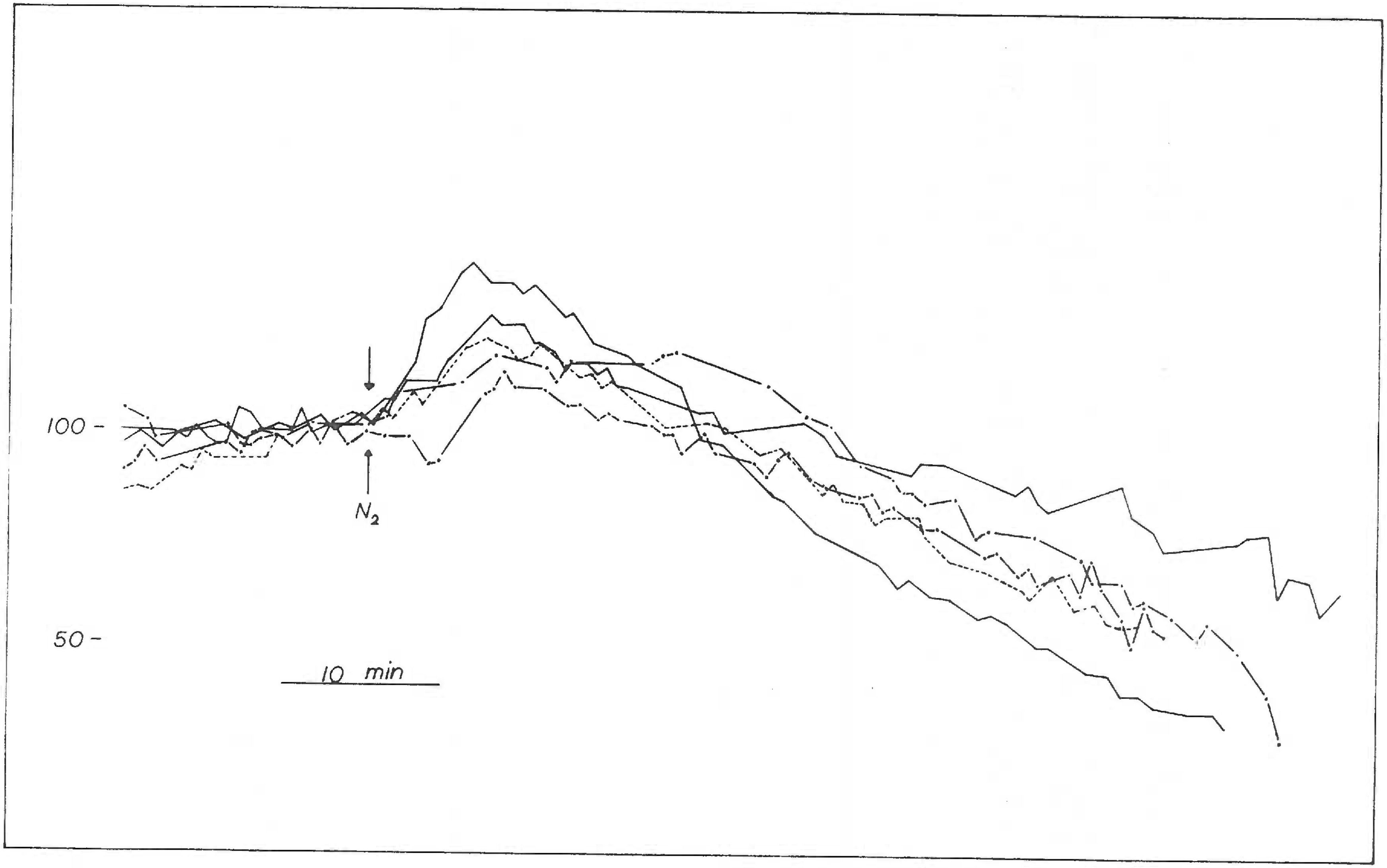
In the preceding paragraphs, it has been shown that depression of discharge from two different neuronal systems accompanies hypoxic episodes. The major difference between the two systems is structural and consists in participation of internuncial activity in the response to dorsal root excitation and the lack of significant internuncial participation in the response to lateral column stimulation. It is, therefore, of interest to inquire whether any differences can be detected in the rate at which the two systems fail during hypoxia episodes. The data in Table VI were tabulated to facilitate comparison of the survival times of lateral column induced discharges with the dorsal root induced reflexes. In all Group I experiments the monosynaptic reflex outlasted that of the dorsal root or polysynaptic reflex. When similar observations were made on spinal cords prepared for micro-electrode studies (Group II) both reflex survival times were significantly longer than they were for Group I experiments. However, in Group II experiments, no consistent difference between monosynaptic and polysynaptic reflex survival was evident. Considering all 10 observations together,

FIGURE 18. Monosynaptic Reflex Magnitude During Hypoxia

Each trace represents the results from five experiments where the magnitude of reflex activity is plotted vs time.

Magnitudes are the integral of spinal nerve discharges presented as % of mean control level. (All experiments from Group I).

Onset of hypoxic period is indicated by the arrows.


100 -
50 -
N$_2$
10 min

there were only two experiments where the polysynaptic reflex outlasted the monosynaptic reflex (see Table VI).

The differential in the survival times of Group I and Group II experiments deserves special attention. Re-examination of the records permits rejection of several possible reasons for this difference. The sequence of the experiments was such that seasonal variations can be ruled out. Similarly, the occurrence of the long and short survival times was such that the development of skill in preparation and manipulation cannot be related to the difference. There were two major differences between the technical procedures involved in preparing the spinal cords. The Group II experiments involved manipulation of the meninges and penetration of the cord substance by the micro-electrode tip. These procedures were not carried out in Group I experiments. It would be highly unlikely that the damage to the preparation resulting from meningeal manipulation and electrode penetration would prolong its survival time. The second major difference involved the amount of time elapsing between the introduction of the isolated cord into the chamber and the initiation of the hypoxic episode. This <u>preparation time</u> for the Group II experiments was invariably greater than that for the Group I experiments because of the technical procedures just mentioned. However, it seemed worthwhile to inquire whether differences in preparation time could be correlated with the differences in survival time.

Accordingly, the relation between preparation time and survival

TABLE VI

MINUTES SURVIVAL TIME FOR MONOSYNAPTIC AND POLYSYNAPTIC REFLEXES
(N=5 PAIRED OBSERVATIONS EACH GROUP)

Spinal Nerve - Group I	Range	Median	Mean	S.D.
Monosynaptic (lat.col.)	42-65	52	51.8	8.6
Polysynaptic (dor.root)	33-48	40	40.4	5.9
Median diff. (mono-poly)= 9 min.				
Spinal Nerve - Group II				
Monosynaptic (lat.col.)	64-115	87	86.3	17.1
Polysynaptic (dor.root)	31-105	75	69.9	27.2
Median diff. (mono-poly)= 0 min.				

time was first determined for the composite of all experiments in which paired observations of mono and polysynaptic responses were made during hypoxia. The resulting linear regression analysis and Kendall rank order correlation are presented in Figure 19. For monosynaptic response the regression coefficient (Pearson product moment) is significant ($p < .005$). For the polysynaptic responses the Kendall rank order correlation was significant to the 0.05 level of rejection. According to these results, the possibility exists that the preparation time constitutes an uncontrolled variable which might influence survival times.

In view of this possibility, other experimental groups have also been subjected to the same kind of analysis. No one experimental group showed any correlation of preparation with survival time. Consequently, two experimental groups were combined to form composite populations and examined for correlation. When this was done, only one population showed any correlation of preparation time with hypoxic survival time (see Fig. 20). This population was composed of polysynaptic reflex data from cords stimulated at 4 and 40 second intervals, and incubated at medium temperatures (17.5-19.5° C). The correlation coefficients are significant to rejection levels in excess of 0.02. The possible meaning of these observations will be considered in the discussion.

DIFFERENTIAL SENSITIVITY OF REFLEX ELEMENTS TO HYPOXIA

In the results presented above, attention has been centered on the amount of motor neuronal discharge initiated by reflex excitation

FIGURE 19. Correlation of Preparation Time with Hypoxic Survival Time

Computer generated plot of data from a population composed of Group I and Group II experiments. Since these two groups contain paired observations of monosynaptic and polysynaptic reflexes, the results from both types are included here.

Monosynaptic reflexes
  Asterisks and solid regression line

Polysynaptic reflexes
  Triangles and dashed regression line

The pertinent statistical data is presented for both types of reflexes. In order of display:

1. Linear regression equation
2. Pearson product moment correlation coefficient
3. t statistic computed from the linear regression coefficient
4. Tail - Kendall rank correlation coefficient

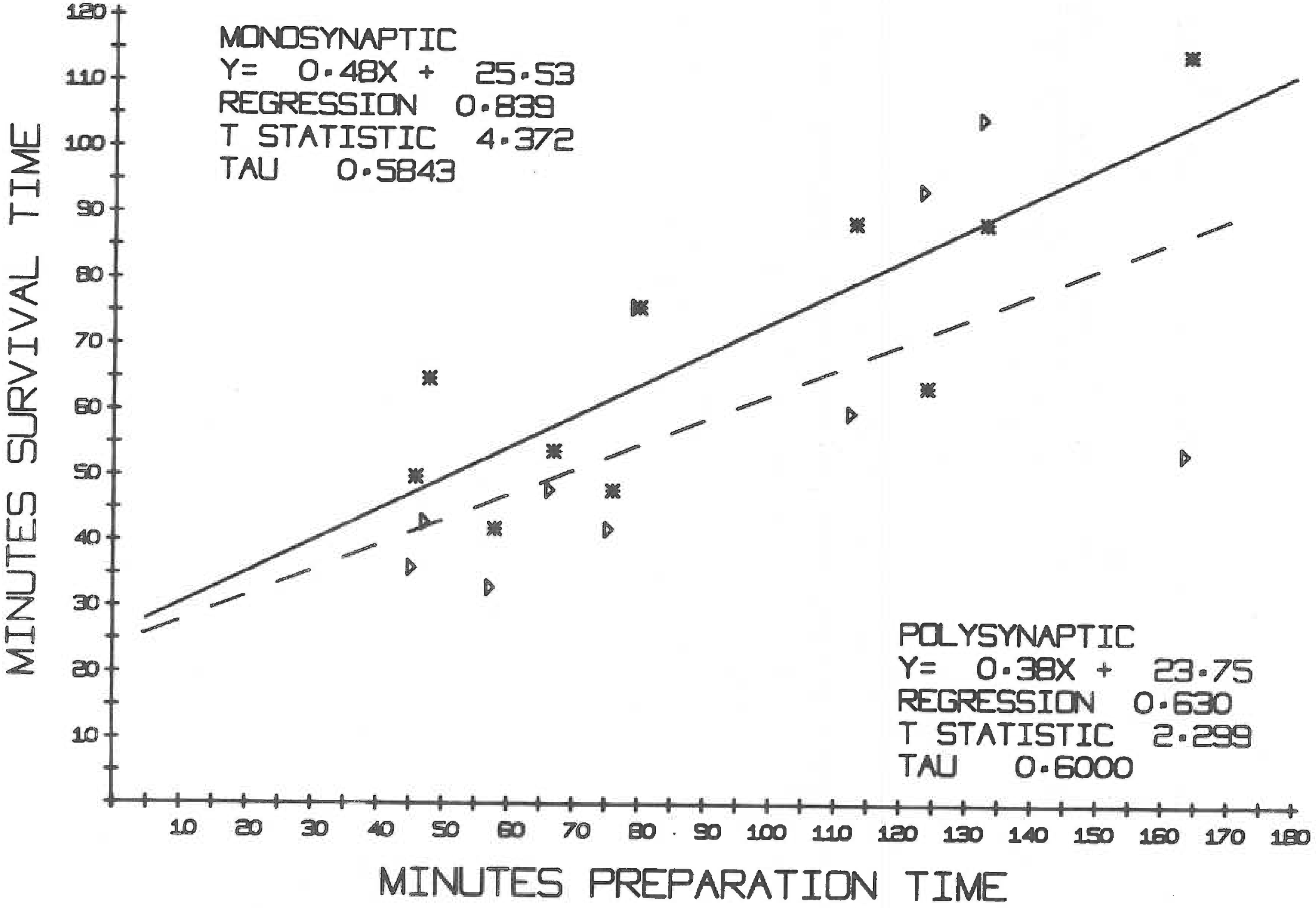
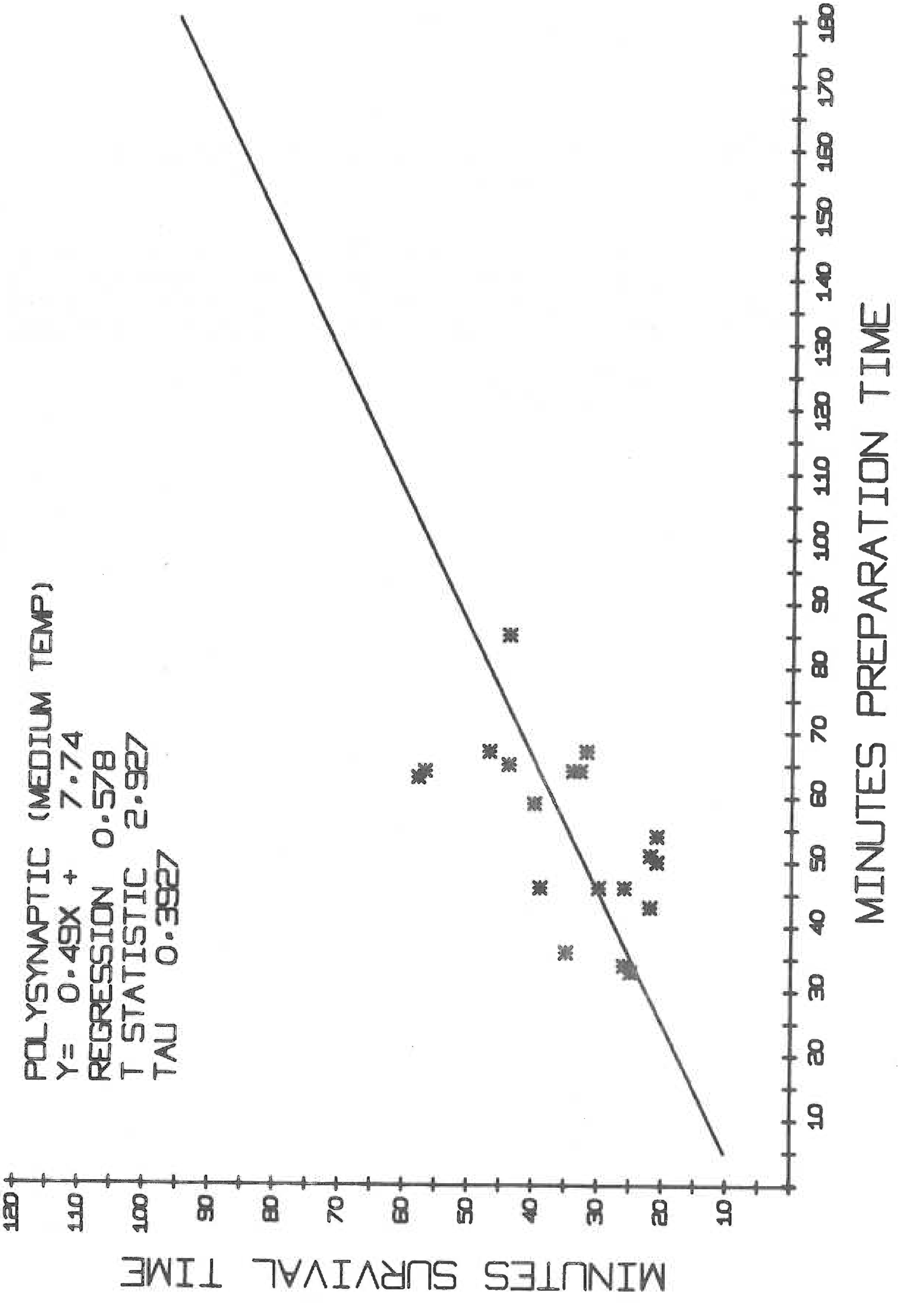

MONOSYNAPTIC
Y= 0·48X + 25·53
REGRESSION 0·839
T STATISTIC 4·372
TAU 0·5843
POLYSYNAPTIC
Y= 0·38X + 23·75
REGRESSION 0·630
T STATISTIC 2·299
TAU 0·6000
MINUTES SURVIVAL TIME
120
110
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
MINUTES PREPARATION TIME

FIGURE 20. Correlation of Preparation Time with Hypoxic Survival Time

Computer generated plot and computations from data taken from all polysynaptic experiments performed at medium environmental temperatures (17.5-19.5° C). This population of data is derived from experiments where the stimulus interval was 4 or 40 seconds.

Statistical data tabulated in the same order as Figure 19.


POLYSYNAPTIC (MEDIUM TEMP)
Y= 0.49X + 7.74
REGRESSION 0.578
T STATISTIC 2.927
TAU 0.3927
MINUTES SURVIVAL TIME
MINUTES PREPARATION TIME
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180

and the changes in this parameter induced by hypoxia. It must be remembered, however, that the discharge of motor neurons is only the final event in a complex chain of processes which transpire in the activated reflex pathway. If real insight into the mechanisms underlying hypoxic failure of neuronal function is to be obtained, an attempt must be made to inquire whether there is a differential sensitivity to lack of oxygen within this set of processes which lead up to motor neuron discharges. The experiments to be described in this section were designed to yield information about these events. The reactions of peripheral axonal components of the reflex arc to oxygen deprivation were examined. In addition, focal potential recordings were used to obtain information about the reactions of presynaptic terminals and of the dendritic and somatic portions of the motor neurons.

Evidence Related to Peripheral Components of the Reflex Arc

The first experiments in this series deal with peripheral elements of the reflex arc. A number of investigators have noted the relative insensitivity of peripheral nerve function to hypoxia (49,50). However, one experiment was performed in a slightly different manner than those in the literature. The effects of hypoxia were observed on a frog sciatic nerve under the identical physical conditions that were used for the spinal cord experiments. During the control period the maximal magnitude of discharge was monitored, but just prior to the start of hypoxia, the stimulus strength was reduced to produce a discharge approximately half-maximal. The magnitude of discharge during the hypoxic period decreased slightly (15%), but at the end of 90 minutes when the stimulus strength was increased, the response returned to the control magnitude. Unlike spinal reflexes, the nerve discharge did not augment during hypoxia and proved to be considerably more resistant than spinal reflexes.

In two experiments intended for lateral column studies, current spread from the stimulating electrodes was sufficient to cause direct excitation of motor neurons. The discharge of directly stimulated motor neurons did not decrease during the period of hypoxia. However, if the stimulus strength was sufficient to cause only 1/2 maximal discharge, the response was seen to augment shortly after the onset of hypoxia. Since peripheral nerve does not augment during hypoxia, response to direct stimulation of motor neurons must be influenced by intraspinal events during hypoxia.

One last piece of evidence to suggest that the extraspinal elements are not the weak links in the reflex pathway is derived from micro-electrode focal potential recordings of antidromically activated motor neurons. When the motor neurons were activated antidromically by stimulation of the spinal nerve, the focal potential changes reveal the activity in the axonal segments as well as that in the soma-dendritic portions of the neurons. Figure 21C, and 22 contain examples of this kind of discharge. The initial positive deflection signals axonal activity prior to antidromic invasion of the motor neuron pool. In all experiments this positive deflection was observed to remain unchanged in amplitude for as long as 135 minutes of hypoxia.

From the above observations, it is apparent that peripheral axons are quite resistant to hypoxia. Reflex failure, therefore, must be caused by failure of elements inside the spinal cord. The next series of experiments was designed to examine intraspinal processes and their susceptibility to hypoxia.

Evidence Related to Central Components of the Reflex Arcs

Focal potential recordings from the motor neuron pool allows one to assess the relative activity of the afferent and the efferent elements of the reflex pathway inside the spinal cord. When lateral column fibers are stimulated a monosynaptic reflex is produced which involves only the afferent bundle of axons and the motor neuron as the elements of the reflex arc. A micro-electrode in the motor neuron pool should be especially sensitive to the terminal portions of the lateral column axons and the motor neuron soma. Figure 21 contains three records from a typical experiment of this kind. Amplitude rather than integral measurements were made on the focal potentials since attempts at integration of micro-electrode signals were unsatisfactory due to noise and high integration rates. The record in part A of Figure 21 contains three traces. The upper trace is the focal potential derived from the micro-electrode. There is an initial positive-

FIGURE 21. Examples of Records from a Micro-electrode Experiment

Part A: Focal potential, spinal nerve potential, and integral in response to lateral column stimulation.

Upper trace - monosynaptic focal potential
p and n are the amplitudes of the positive and negative waves, respectively, of the presynaptic focal potential.
m - amplitude of the postsynaptic focal potential

Middle trace - integral of the spinal nerve discharge - (int. - amplitude of the monosynaptic portion of the integral)

Part B: Focal potential, spinal nerve potential, and integral in response to dorsal root stimulation.

Upper trace - polysynaptic focal potential
p - amplitude of the second p wave
m - maximum amplitude of the postsynaptic focal potential

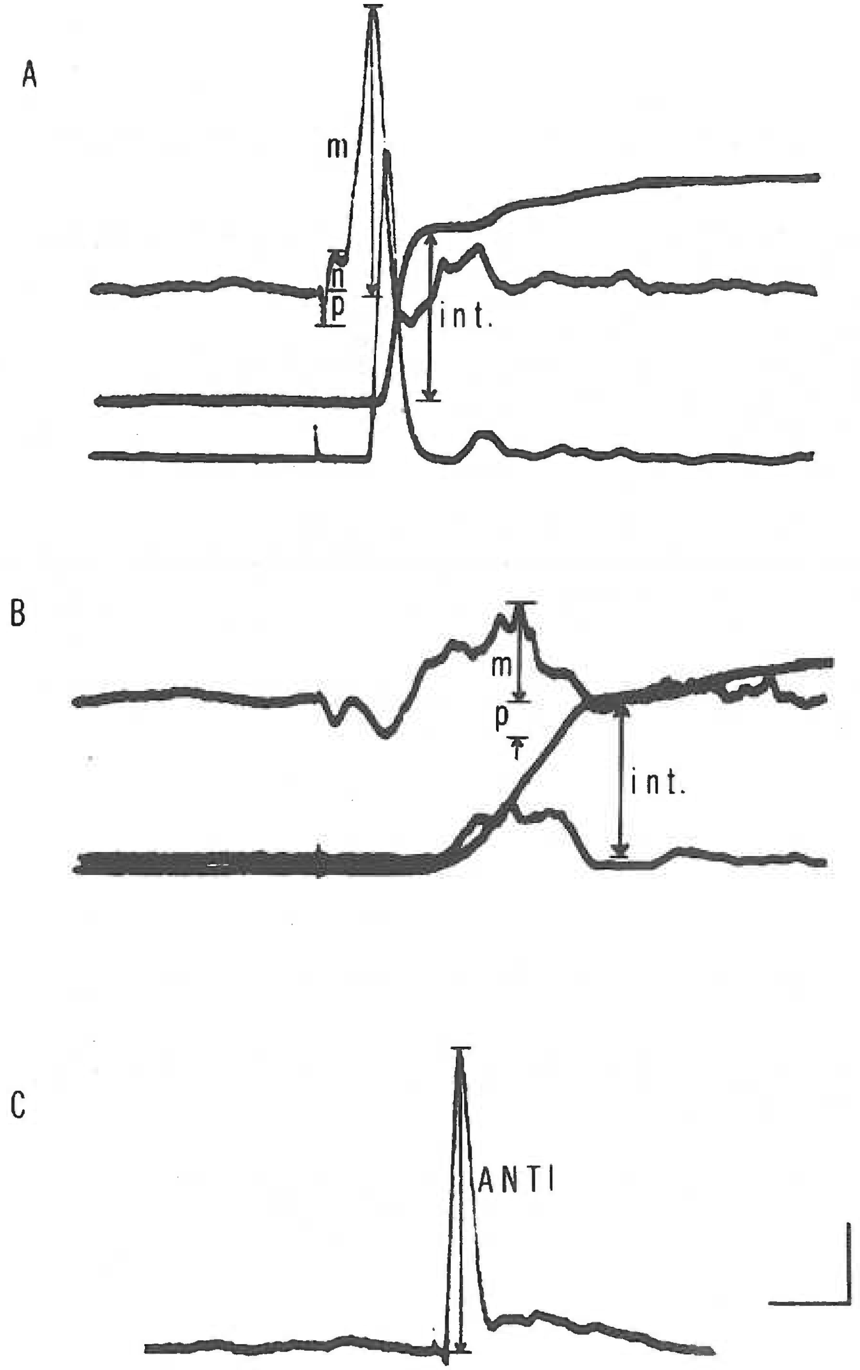
Middle trace - spinal nerve discharge

Lower trace - integral of the spinal nerve discharge

Part C: Antidromic focal potential. Anti is the amplitude measured from base line.

Amplitude calibrations: Focal potential - 2.5 mv
Spinal nerve - 400 μv
Integral - 5 v-msec

Duration = 5 msec/div.


A
m
n
p
int.
B
m
p
int.
C
ANTI

negative deflection (p) wave which is the electrotonic signal of the oncoming lateral column volley. The n wave signals the arrival of the volley into the region of the motor neuron pool. The n wave is almost immediately followed by a large negative potential (m wave) which represents postsynaptic activity. It can be seen that there are potential fluctuations following the m wave which probably represent activity via polysynaptic pathways.

The lower and middle traces represent the spinal nerve discharge and integral respectively. The onset of spinal nerve discharge is about 1 msec. after the beginning of the m wave. Since the m wave is produced at least in part by the discharge of motor neuron somata, the conduction velocity of the fastest motor neurons was about 30 meters per second in this experiment.

The upper trace in part B of Figure 21 is the focal potential response to dorsal root stimulation. The polysynaptic focal potential is considerably more complicated than the monosynaptic. There are two positive waves (p) and a diphasic negative wave (m). The two lower traces are the spinal nerve potential and its integral.

Part C of Figure 21 is an antidromic focal potential produced by stimulation of the proximal spinal nerve. It is generated principally by discharge of neuronal somata.. As previously described, the small initial positive potential is an electrotonically conducted signal of axonal discharge prior to the invasion of the somata.

Measurements made from focal potentials produced during hypoxia should allow one to test the hypothesis that hypoxic reflex failure occurs because of conduction failure in the fine terminal branches of the afferent axon. If this hypothesis is true, one might expect that the p wave of the monosynaptic focal potential would not decrease and probably would increase during reflex failure since axons are relatively insensitive to hypoxia. The n wave which should be principally generated by lateral column collaterals and fine terminal branches in the motor neuron pool should decrease as the reflex disappears. The postsynaptic potential (m wave) should also decrease during failure since it is principally generated by synaptically activated motor neurons and therefore is contingent on activity represented by the n wave. Furthermore, if failure is truly presynaptic in origin, the antidromic focal potential should not markedly decrease during hypoxia, as this is a test of motor neuron excitability. With the above considerations in mind, the reader should examine Figure 22 which contains records of the control, hypoxic, and recovery periods of a typical experiment. After 155 minutes of hypoxia, the lateral column focal potential showed considerable decrement in postsynaptic response (m wave). However, the p wave was reduced in amplitude and was even more reduced in amplitude than the n wave. The whole presynaptic signal was decremented. At the same time, the polysynaptic focal potential (dorsal root) was unchanged with respect to the initial positive deflection, but the later negativity as in the lateral column response was considerably reduced. The fact that

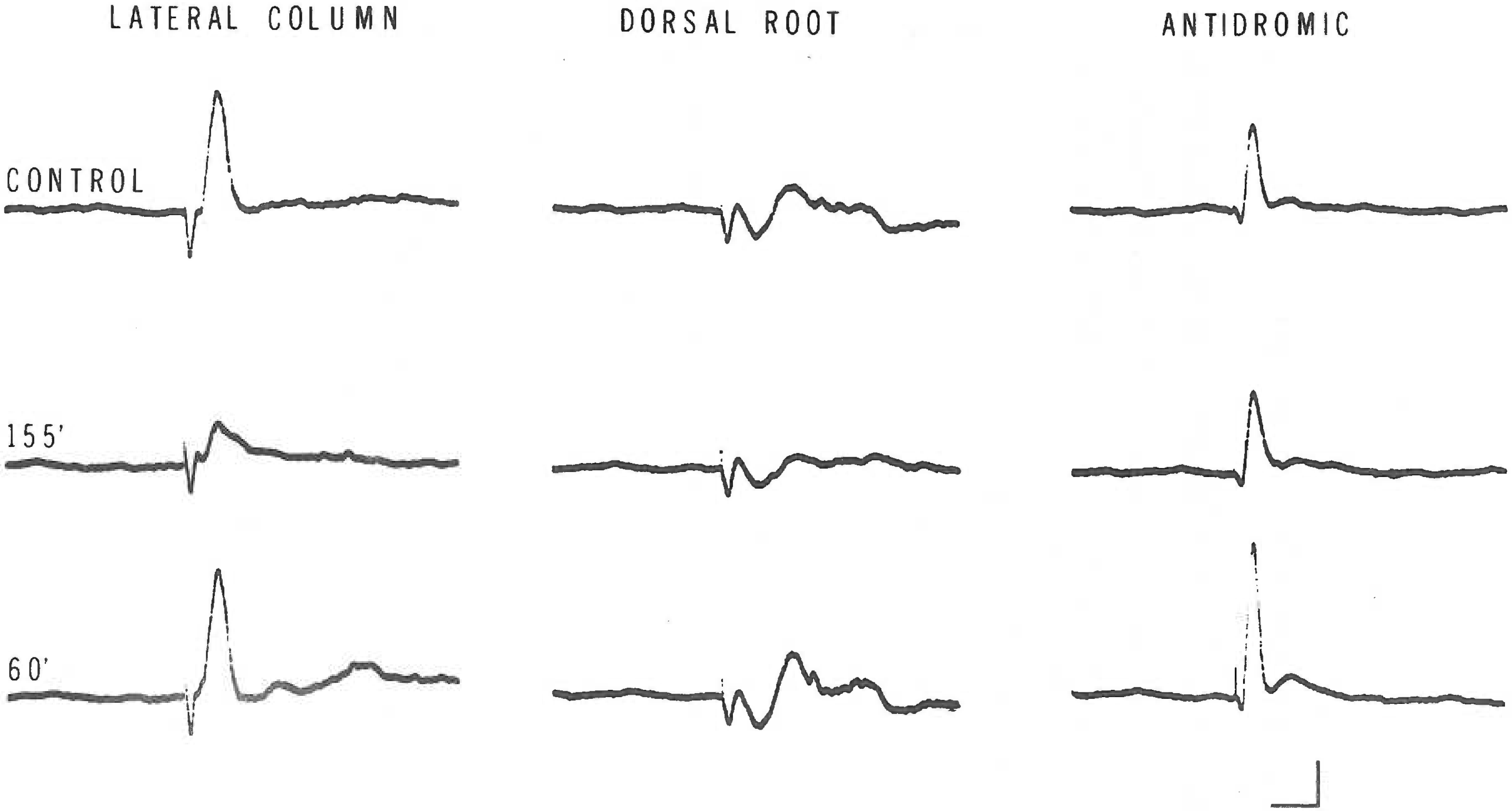
FIGURE 22. Focal Potentials During Hypoxia

Upper row - focal potential records during the control period.

Middle row - focal potentials after 155 minutes of hypoxia

Lower row - focal potentials after 60 minutes of recovery in 95% O_2

Calibration mark - 2.5 mv and 5 msec.


LATERAL COLUMN
DORSAL ROOT
ANTIDROMIC
CONTROL
155'
60'

the motor neuron soma was still excitable to invasion from antidromic propagation after 155 minutes of hypoxia is demonstrated by the antidromic focal potential which was essentially unchanged with respect to the control period.

After 60 minutes of recovery under aerobic conditions, the monosynaptic and polysynaptic focal potentials were quite similar to control responses at the start of the experiment. The antidromic focal responses, however, appeared somewhat different from responses during the control period in that it was more synchronous and is followed by a more prominent late negative potential.

It would be of interest to compare focal potentials with potentials recorded from the spinal nerve during hypoxia. Figure 23 is presented for this purpose. After 120 minutes of hypoxia changes in focal potential records occurred in the same fashion as in the experiment described from Figure 22. However, in this experiment, recovery was less complete and the antidromic focal potential was quite similar to that during the control period. During hypoxia, the monosynaptic spinal nerve potentials decreased in magnitude without any initial augmentation. The polysynaptic spinal nerve potential decreased gradually after augmentation and reached 50% of control level before the lateral column response. As mentioned previously, the 50% survival time of the spinal nerve potential in this and other micro-electrode experiments is considerably longer than in experiments not prepared for micro-electrode measurements.

FIGURE 23. Reflex Activity in One Experiment from Spinal Nerve and Focal Recording Electrodes

Upper row - records from control period

Middle row - records after 120 minutes of hypoxia

Lower row - records after 65 minutes of recovery in 95% O_2

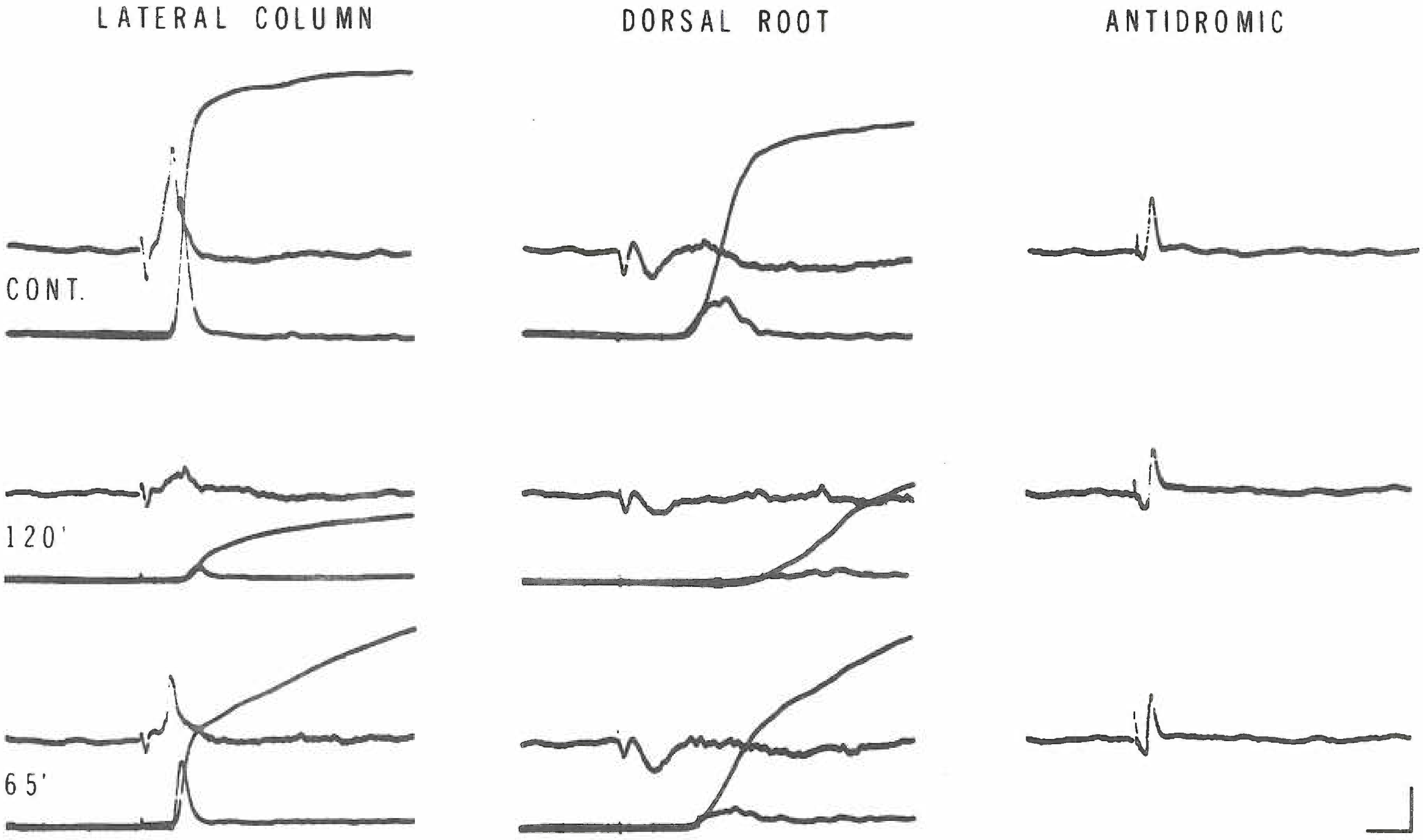
Upper trace - focal potential in each record

Lower trace - spinal nerve potential and its integral

(Antidromic responses - focal potential only)

Calibration mark
  Amplitude
    Focal potential - 2.5 mv
    Nerve potential - 1 μv
    Integral - 5 volt-msec

Duration - 5 msec.


LATERAL COLUMN
DORSAL ROOT
ANTIDROMIC
CONT.
120'
65'

The following characteristic changes during hypoxia were consistently observed in the focal potential studies:

> 1. The presynaptic signal decreased in amplitude with respect to the positive and negative portions of the signal in all experiments.
>
> 2. The antidromic focal potential usually increased somewhat in amplitude during hypoxia. In one experiment where the antidromic focal potential markedly decreased, it was 68% and 40% of control when the spinal nerve potential was 50% and 18% respectively.
>
> 3. There was only one instance of complete reflex failure within 185 minutes of hypoxia out of seven experiments. In this case, the antidromic focal potential was 98% of control and the presynaptic positivity of the monosynaptic focal potential was 60% of control.

These results indicate that the motor neuron soma is the least sensitive of the intraspinal elements to hypoxia. They also indicate that reflex failure is synaptic or presynaptic in nature but do not critically establish that the fine terminal branches of the afferent axon are the most sensitive elements in the reflex path to hypoxia.

WHY DOES FAILURE OCCUR?

The previous sections have described results of experiments designed to uncover the site of reflex failure. Certain elements, notably the motor neuron soma and peripheral nerve, have been shown to be quite resistant to the effects of hypoxia. However, these experiments shed no light upon the questions of why or how reflex failure is caused.

Metabolic Correlations

Previous investigators have observed by intracellular recording that spinal neurons depolarize during hypoxia (41,56,61,62,69). When these neurons depolarize to a level above threshold potential they will become inexcitable and fail to initiate an action potential. Though this failure may be the direct result of a passive physical process, the primary disturbance of hypoxia is metabolic in nature. Since hypoxia results in reduction of oxidative metabolism, then reflex activity may be dependent upon high energy intermediates whose production is curtailed by hypoxia. It is likely that the unstimulated spinal cord requires these metabolic intermediates in order to maintain its excitability. Furthermore, there is ample evidence that nervous activity also requires energy in order to restore the neurons to their initial pre-discharge conditions [(14), page 72]. At the onset of hypoxia, it is hypothesized that energy metabolites will not be produced at a rate sufficient to meet the demands of the tissue. Thus, when the limited stores of these intermediates are rate limiting, neuronal excitability should begin to fail. If the processes underlying the maintenance of reflex discharge in this preparation requires a significant proportion of the total energy requirements of the tissue then reflex activity should fail sooner when the preparation is stimulated more often. This hypothesis is diagrammed in Figure 24 A. If pathway A is the resting metabolic energy drain, and B the added drain due to nervous activity, then it is

FIGURE 24. Metabolic Aspects of Hypoxic Reflex Failure

Schematic diagram - a greatly simplified representation of the metabolic energy flow in the spinal cord. Pathway A represents energy demand by the tissue at rest. Pathway B represents the added demand of nervous activity. The hypothesis assumes that reflex survival time is inversely proportional to total metabolic rate (A+B).

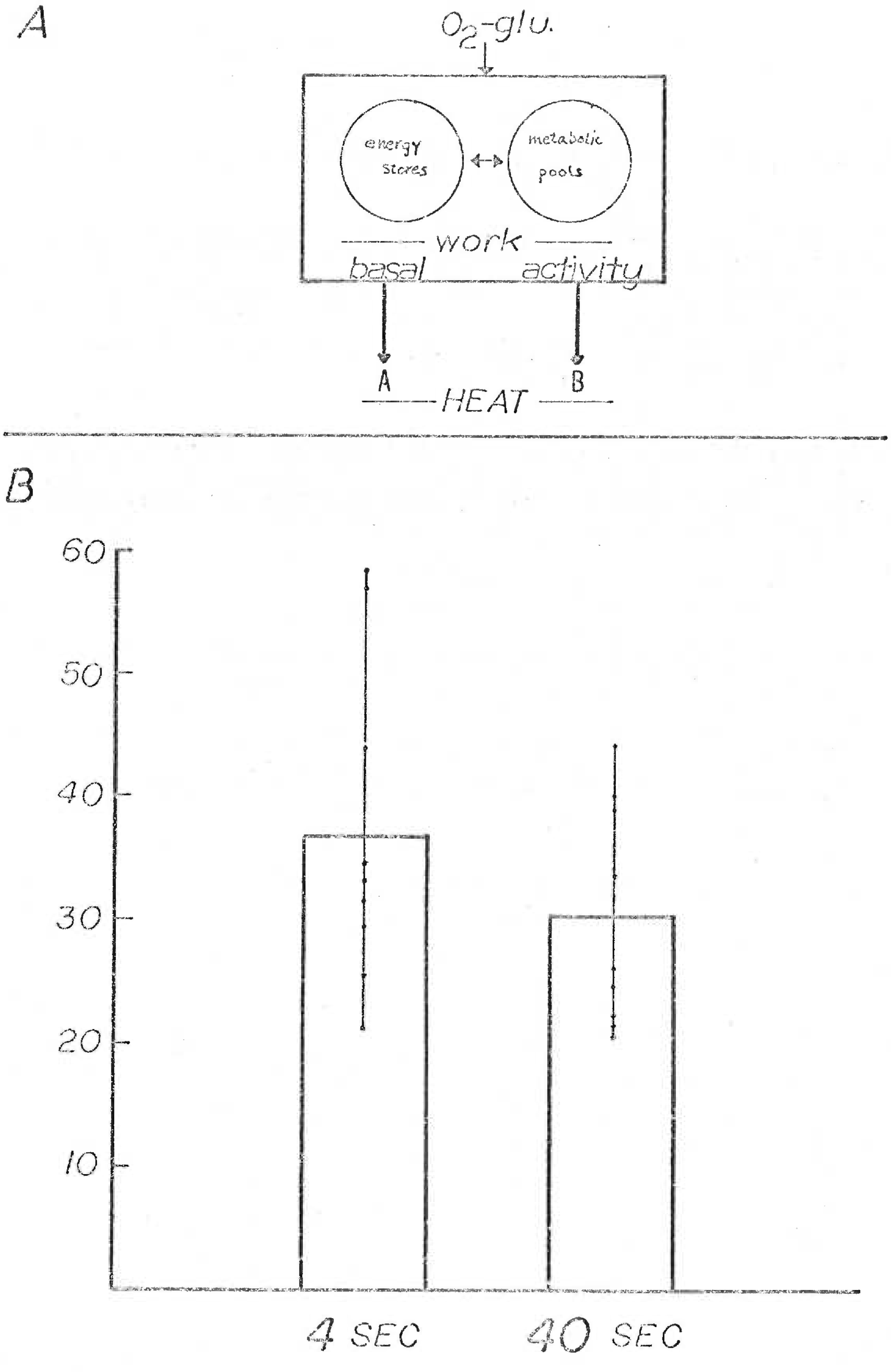
Histogram - The results of experiments where the energy demand in pathway B (above) was altered. The polysynaptic survival times of two populations of spinal cords handled in the same way except for stimulus interval.

Abscissa - stimulus interval
Ordinate - survival time minutes

Data points are plotted to show the range of values. The histogram bars extend to the arithmetic mean of the group.

Mean survival time 4 second intervals - 38.1 minutes

Mean survival time 40 second intervals - 30.2 minutes


A
O2-glu.
energy stores
metabolic pools
work
basal
activity
A
B
HEAT
B
60
50
40
30
20
10
4 SEC
40 SEC

hypothesized that the survival time of reflex activity in the cord will be inversely proportional to the magnitude of the quantity (A+B).

To test this hypothesis, one may vary the magnitude of B- the energy drain due to activity. Accordingly, survival times of cords stimulated at two rates, an order of magnitude different, are compared in Figure 24 B. There is no statistical significance in the difference of the mean or median survival times between the two stimulus rates.

The corollary of the above hypothesis is that: The survival times of spinal cords should be inversely proportional to the resting metabolic rate (pathway A). One method of varying this element would be to adjust the environmental temperature of the spinal cord. The results of these experiments are shown in Figure 25. The survival time was indeed found to be inversely proportional to the environment temperature ($p < 0.001$). The Q_{10} is 2.5 (10°-20° C) which is typical of biological systems rather than physical processes.

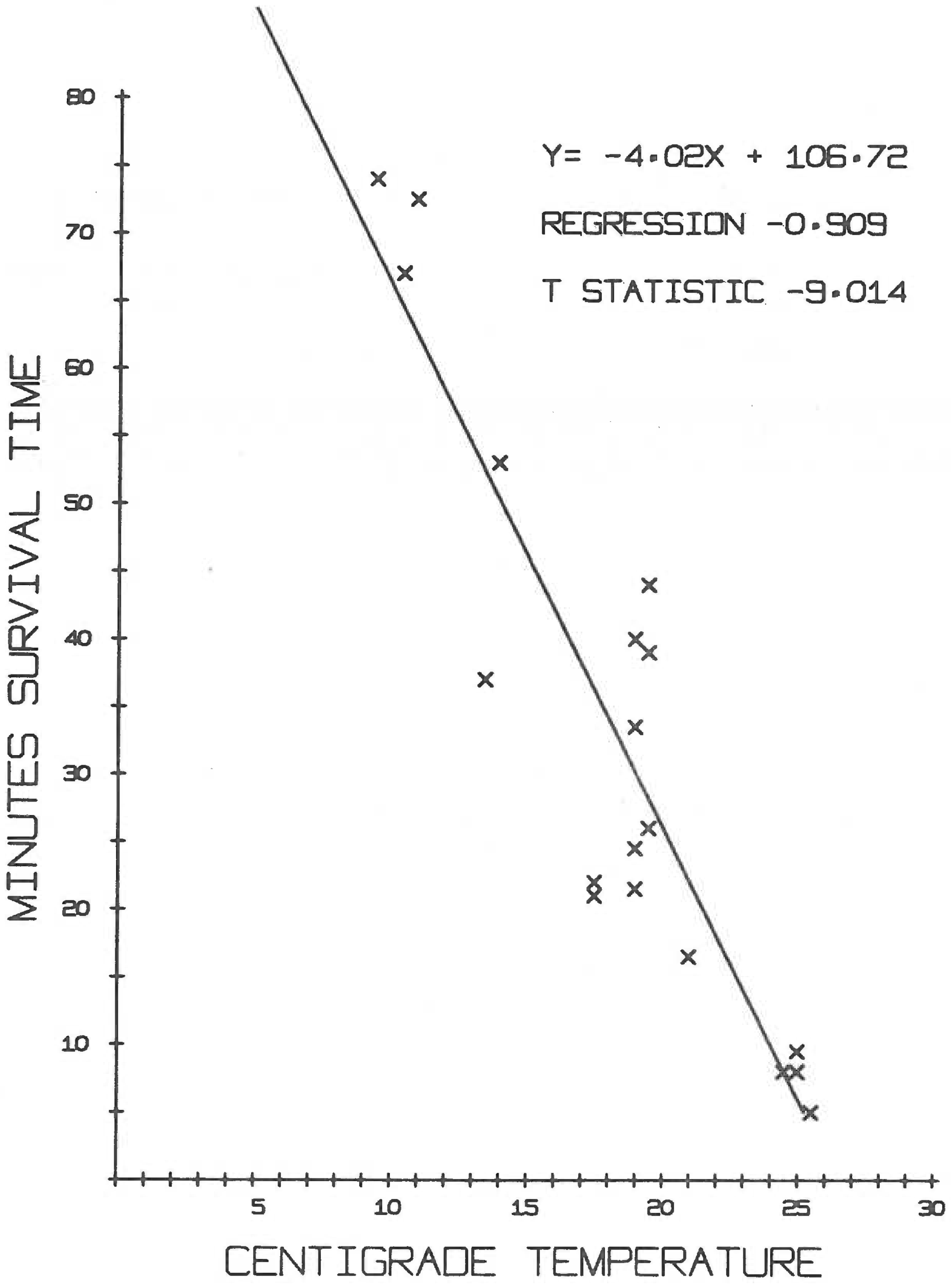
One can conclude, therefore, that most of the metabolic requirements of the spinal cord exist in the resting state and are not greatly increased by causing the reflex system to generate activity. Symbolically the hypothesis may be expressed in the following form: Energy costs are a function of the magnitude of A+B where $A \ggg B$. The experiments show that hypoxia and survival times are related metabolically but do not suggest the mechanism by

FIGURE 25. Polysynaptic Reflex Survival Times vs Temperature

A computer generated plot of the data including a linear regression analysis.

The statistical summary in order displayed:

1. Linear regression equation
2. Regression coefficient (Pearson product moment)
3. t statistic to test significance of the regression coefficient ($p < 0.001$)



Y= -4.02X + 106.72
REGRESSION -0.909
T STATISTIC -9.014
MINUTES SURVIVAL TIME
80
70
60
50
40
30
20
10
5
10
15
20
25
30
CENTIGRADE TEMPERATURE

which hypoxia causes nervous tissue to become inexcitable. Based upon the evidence in the literature, a reasonable hypothesis would be that hypoxia primarily results in failure of the active ion transport that sustains the transmembrane ion gradients necessary for excitability.

Ouabain vs Hypoxia

In order to test this hypothesis it would be necessary to specifically inhibit the sodium pump. Ouabain has been postulated to act as a specific inhibitor of the sodium pump in a number of tissues [(113), page 305]. The evidence is quite good for several tissues of the frog including peripheral nerve. Accordingly, some preliminary experiments were devised to gain confidence that the spinal cord tissue was sensitive to ouabain in a manner similar to other nervous tissues discussed in the literature. It has been demonstrated that the frog spinal cord contains a Na^+, K^+ activated ATP'ase which can be inhibited by ouabain (D. Reed and D. McAfee unpublished results). This is a necessary condition which must be met before it is possible to postulate that ouabain specifically inhibits the Na^+ pump. A second manifestation of sodium pump inhibition that has been accepted by many is the reduction in O_2 consumption which accompanies the action of ouabain. Preliminary tests of this aspect of ouabain action have been carried out using spinal cord tissue brei. In all trials (four experiments) ouabain consistently reduced oxygen consumption to about 70% of its original value. Addition of cyanide completely inhibited all oxygen uptake. Similar results have been observed with cerebral cortex (118). The fact that ouabain inhibits oxygen consumption suggest that the energy requiring sodium pump in the frog spinal cord is sensitive to ouabain. The total amount of inhibition is equivalent to that seen in cerebral cortex (118).

Since it has been hypothesized that hypoxia primarily inhibits the sodium pump, ouabain should also cause reflex failure and do so with a pattern similar to hypoxia. Figure 26 shows focal potential and spinal nerve potential recording from a preparation treated with ouabain. The experiments were carried out in a manner identical to the hypoxia experiments except that the stopcock was turned to admit ouabain oxygenated Ringer instead of nitrogenated Ringer. After 50 minutes in 25 micromolar ouabain Ringer, reflex activity completely disappeared. Furthermore, the antidromic signal contained evidence of only axonal activity (p wave) whereas the soma dendritic invasion was lost. These results are in sharp contrast to results from hypoxia experiments where the antidromic signal never decreased before or to the same extent as the orthodromic. The presynaptic portion of the monosynaptic focal potential also completely disappeared under ouabain. However, in four experiments it disappeared 40-120 seconds after the antidromic signal. After 30 minutes of simple oxygenated Ringer, the reflex activity recovered partially. Another ouabain episode resulted in complete failure within 20 minutes. One singularly startling result is the

FIGURE 26. Effects of Ouabain on Reflex Activity

Reflex activity in response to lateral column (L.C.) stimulation, dorsal root (D.R.) stimulation, and antidromic (anti) stimulation.

First row - control records
Second row - after 50 minutes in ouabain Ringers
Third row - recovery after 30 minutes in ouabain free Ringer
Fourth row - 20 minutes after return to the ouabain Ringer

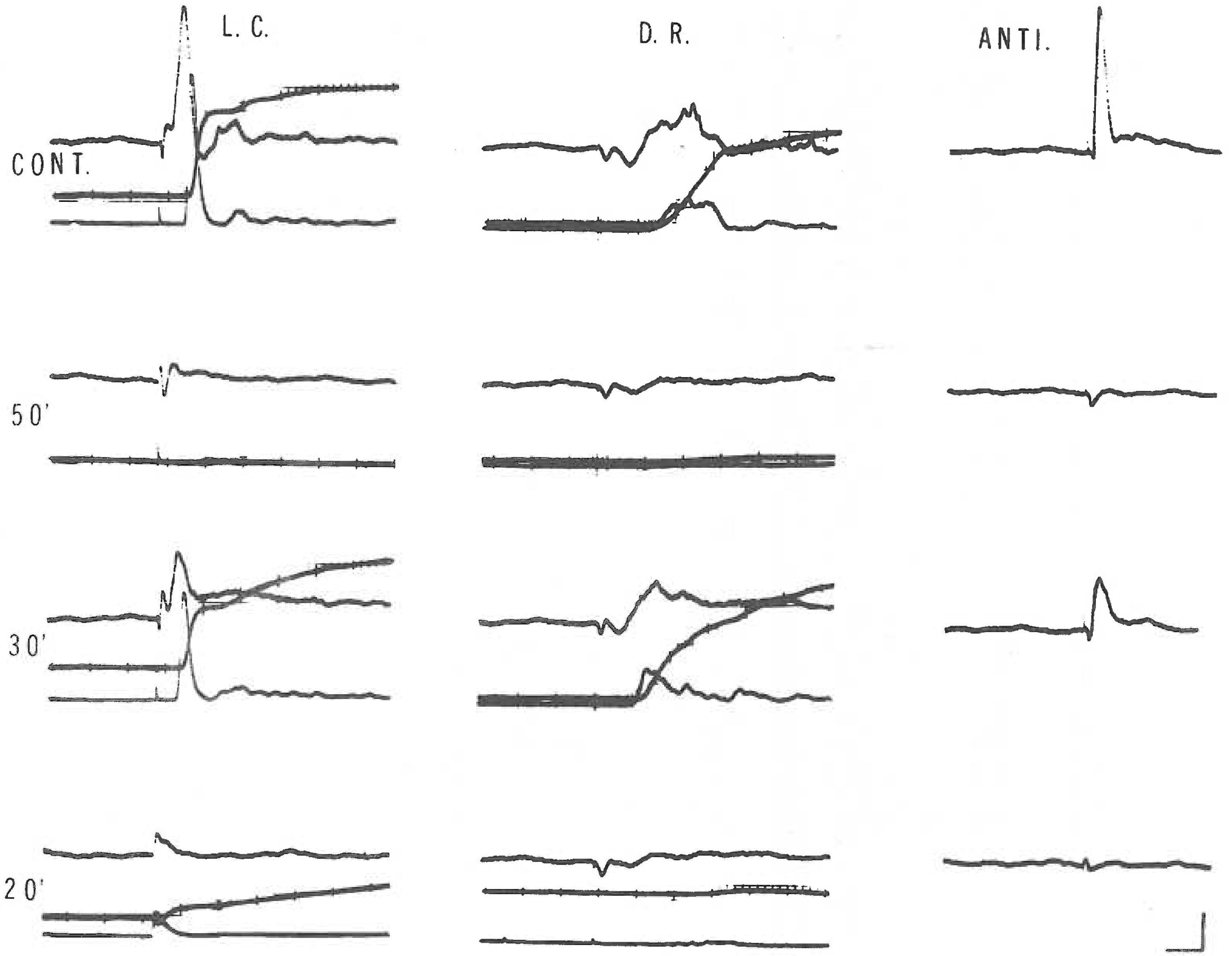
Upper trace represents the focal potential activity

Lower traces are from spinal nerve discharges and its integral. These portions of the records have been deleted from the antidromic records.

Calibration marks

Amplitude
Focal potential - 2.5 mv
Spinal nerve - 400 µv
Integral - 5 volt-msec.

Duration - 5 msec.


L. C.
D. R.
ANTI.
CONT.
50'
30'
20'

pattern of reflex failure during ouabain poisoning. Figure 27 contains records of the various parameters of reflex activity. Failure is not by simple decay, but is an abrupt change in magnitude of response to zero, after which there is temporary recovery followed by a second complete failure of reflex activity. Evidence of this "oscillatory" pattern of reflex failure was seen in all experiments with ouabain and never seen with hypoxia. The survival time of reflex activity to ouabain poisoning is a function of the concentration of ouabain. The dose response relationship can be seen in the graph in Figure 28 plotted to compare survival time and ouabain concentration.

Comparison of ouabain records in Figure 26 with those taken during hypoxia (Fig. 22 and 23) illustrate the difference in the pattern of failure in the various elements of the reflex path. When Figure 27 is compared to Figure 15 and 18, the differences in the pattern of failure of spinal nerve discharge between hypoxia and ouabain become apparent. The significant features of ouabain induced reflex failure as they contrast to hypoxia may be summarized as follows:

1. Reflex failure is periodic
2. Reflex failure is rapid and complete
3. Antidromic excitability does not extend beyond post-synaptic failure
4. Antidromic signals do not outlast presynaptic signals

The discussion section will dwell in detail upon these differences and perhaps offer some meaningful conclusions concerning the mechanisms of hypoxic reflex failure.

FIGURE 27. Patterns of Reflex Failure Under Ouabain

Magnitude of monosynaptic reflex activity as determined by integration of the discharge in the spinal nerve. Magnitude plotted as percent of mean control level vs time. Three different experiments represented here.

Arrows denote time of ouabain experimental period. Recovery from ouabain inhibition shown for curve with dotted line. The O_2 indicates washout with ouabain free oxygenated Ringer.

Solid line - 100 μmolar ouabain oxygenated Ringer during experimental period.

Dashed line - 75 μmolar ouabain in oxygenated Ringer.

Dotted line - 25 μmolar ouabain oxygenated Ringer. Ouabain washed out after about 55 minutes duration of experimental period.

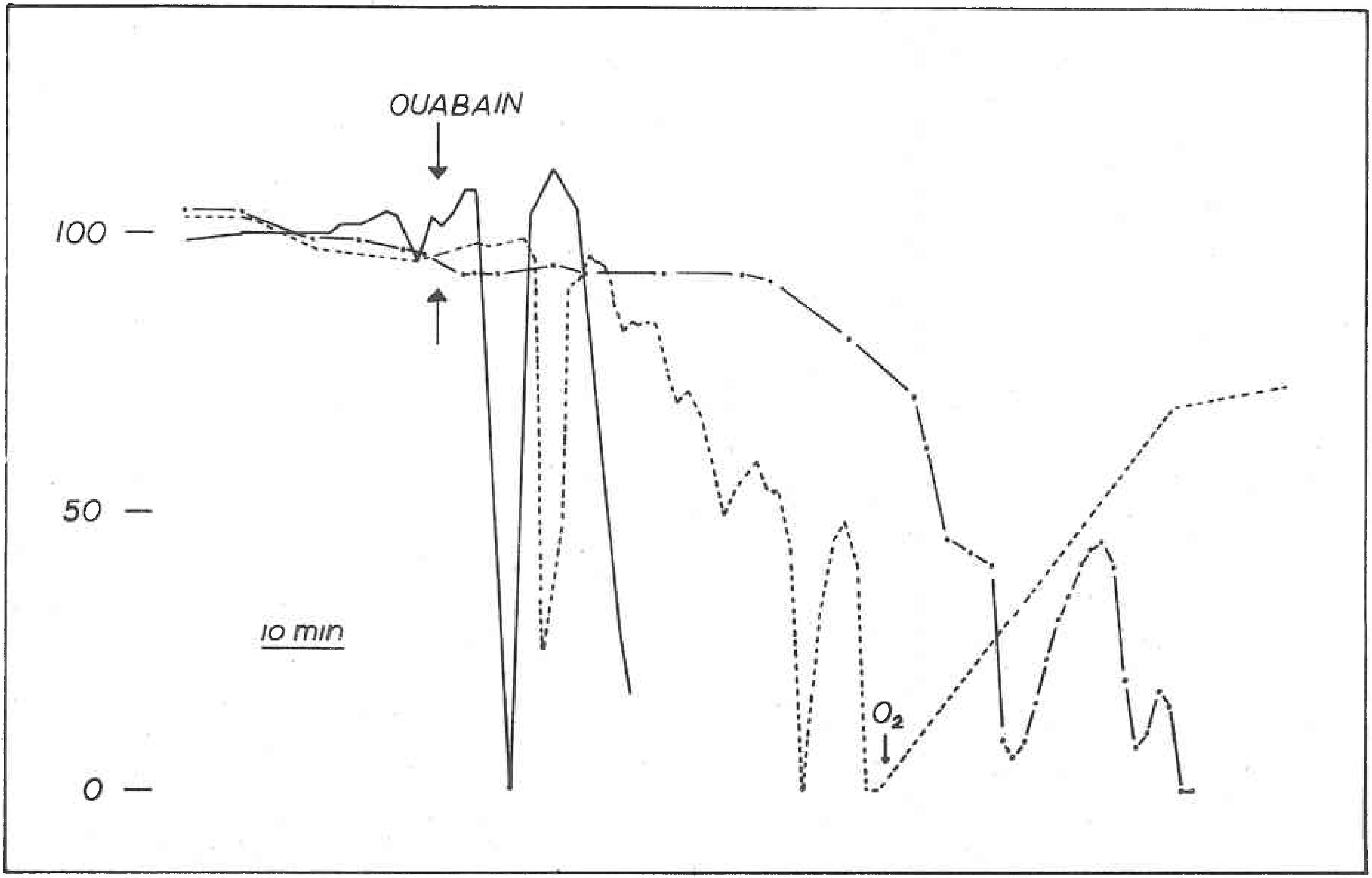
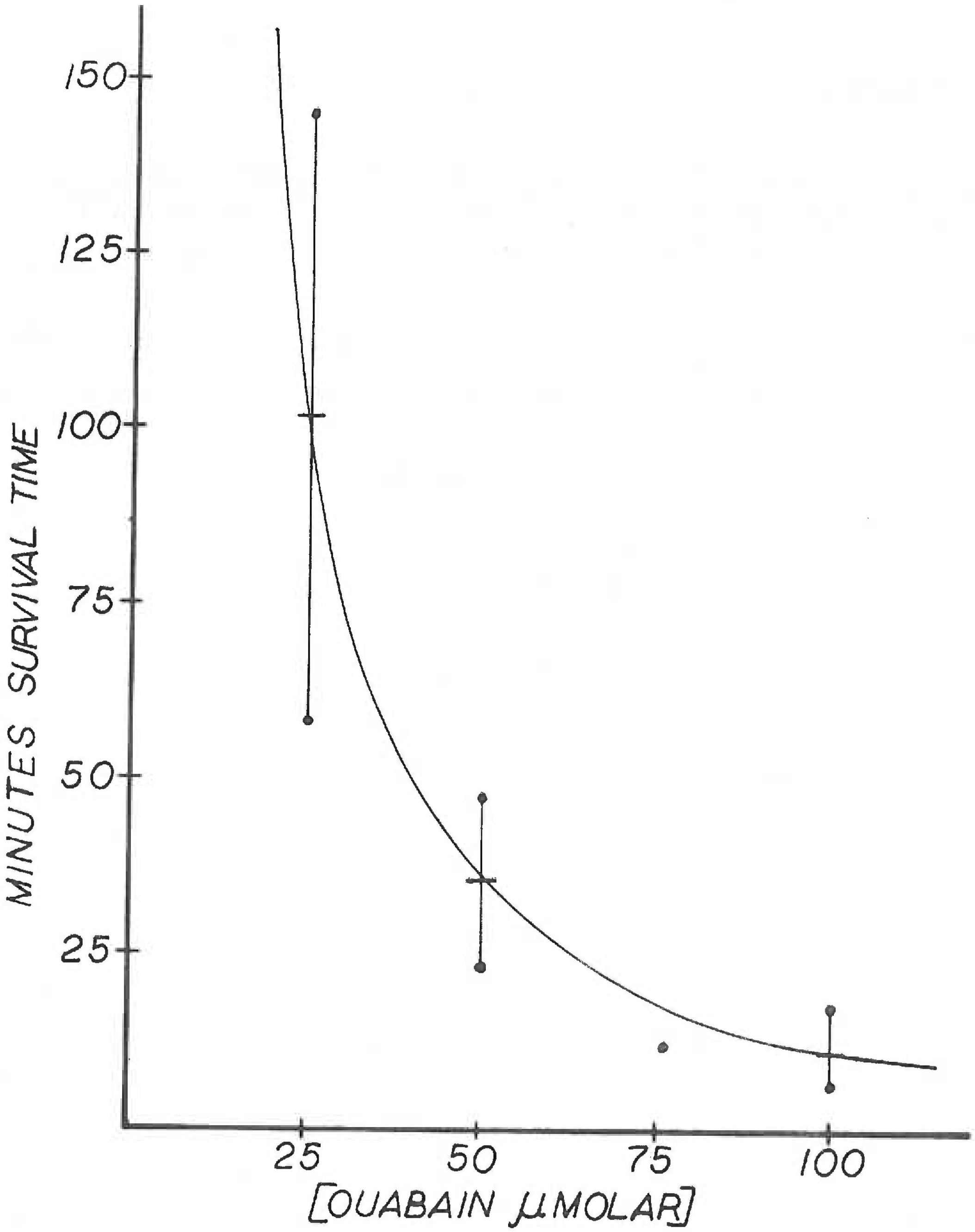

OUABAIN
100
50
0
10 min
O_2

FIGURE 28. Dose Response Relationship

Ouabain μ molar concentration vs survival time of monosynaptic discharge in spinal nerve. (Survival time=time to reach 50% of control magnitude).


150
125
100
75
50
25
MINUTES SURVIVAL TIME
25
50
75
100
[OUABAIN μMOLAR]

DISCUSSION

The experiments which have been described in the previous section were directed toward obtaining further insight into the metabolic activities of nervous tissue by studying the effects of hypoxia on a relatively simple reflex system. In order to achieve this goal it was necessary first to become familiar with the reflex behavior of the chosen preparation and to devise a method of estimating the amount of nervous activity induced by various stimuli. Given predictable behavior capable of being described in quantitative terms, it became possible to examine the influence of metabolic manipulations under conditions which would permit more meaningful interpretations of the results. The results obtained from these experiments will be discussed in relation to three basic questions:

1. What was the basis for selection of criteria for functional measurements?
2. What are the implications of the pattern of hypoxic reflex failure?
3. What might be the mechanisms by which hypoxia causes reflex failure?

In the course of this discussion, the current hypotheses of the mechanisms of reflex failure under hypoxia will be reviewed and related to the results of this study. An attempt will be made to demonstrate that the use of an isolated reflex system in a quantitative fashion provides advantages over other CNS preparations when correlations are sought between neurophysiologic and molecular aspects of function.

CRITERIA FOR FUNCTIONAL MEASUREMENTS

Quantity of Reflex Discharge

The first important criterion was the need for a measure of reflexly initiated discharge from a population of motor neurons. Prior to the development of satisfactory techniques for electrophysiological recording, early investigators used the tension developed in a suitable muscle as a measure of reflex magnitude. However, muscle tension provides only an indirect, non-linear measure of discharge which has unpredictable sensitivity. Thus, quantitative estimates of discharge are difficult or impossible to achieve. The amplitude of reflex discharge has also been used to measure reflex magnitude (54,55,56).

There are several reasons why the measurement of the integral provides more accurate measure of the magnitude of the induced discharge than does amplitude. In contrast to amplitude measures, the integral of nervous activity is not affected by changes in the synchrony of discharge, but only by changes in the total amount of electrical potential developed by the nerve. Unless the voltage-time curve of the discharge is symmetrical about its peak and changes in amplitude are not associated with changes in duration, amplitude will not be a measure of the amount of nerve discharge.

There are three sources of potential that can contribute to the magnitude of the integral-action potentials, electrotonic potentials, and noise. Since electrotonic potentials do not represent discharge, they must be considered more carefully. Large electrotonic potentials were shown to be present when the recording electrodes were placed on the ventral root. Others (147,148) have presented convincing evidence that the source of this potential is the electrotonic conduction along the ventral roots of summated EPSP's generated in the motor neuron cell bodies. Since it was desired to record only potentials from discharge beneath the electrodes, these EPSP's represent a source of unwanted signal. Moving the recording electrodes distally to the extreme end of the spinal nerve caused an 80% reduction in the magnitude of the integrated potentials. One can calculate a length constant of 3.5 mm for a 10 μ diameter myelinated fiber from the constants and formulae in part I of the introduction. This calculated length constant is an overestimate since it does not take into account the shorting effects of the low resistance membrane at the nodes. Recording from the distal spinal nerve resulted in an increased conduction distance of about 15 mm or almost five length constants. Spinal nerve potentials, therefore, should contain a negligible electrotonic component. Consequently, the integral of the spinal nerve potentials as opposed to that of the ventral root should represent the best estimate of the total amount of discharge generated by motor neurons as the result of synaptic activation.

The integrated spinal nerve potentials do not allow one to assess the frequency of discharge. In fact, a neuron that discharges most often will contribute more to the integral than one which discharges only once. Therefore, the integral cannot be a measure of the number of elements discharging, but only of the total number of discharges. Because the efferent elements of spinal nerves are axons of only motor neurons, spinal nerve potentials will contain no potentials from internuncials. Induction of reflex activity, furthermore, will invariably cause discharge of motor neurons whose axons exit from adjacent spinal levels. Thus, the spinal nerve integral is a measure of motor neuron discharge from one spinal level only. The integral of the spinal nerve discharge therefore yields information only about the behavior of the motor neurons contributing to the spinal nerve on the electrodes and cannot be regarded as an index of the total amount of impulse activity in the preparation.

It is difficult to reproduce the recording conditions from experiment to experiment and differences in these conditions have contributed to the variability or variations seen in integrated discharge magnitudes tabulated in Tables III, IV, V. However, standardization of technique reduced the variability between successive experiments to minimum and as a result it was possible to make some meaningful comparisons of magnitude of reflex activity between different experimental groups. For instance, the magnitude of the polysynaptic reflex at low temperatures (9-14° C) was significantly greater than at higher temperatures (15-25° C). Other investigators have reported this phenomenon (151,152,153). Reflexly induced muscle tension was observed to increase during cooling of the frog spinal cord (153). However, in the most recent paper which contributes information in this area, measures of ventral root discharge amplitude were used as a quantitative measure of reflex activity. Tebecis and Phillis (151) reported large changes in reflex "magnitude" when the temperature of the hemisected cord was varied between 0 and 30° C. These results are therefore invalid as far as magnitude of reflex discharge is concerned, but are valid with respect to the change in the synchrony of discharge which increased markedly as the temperature fell below 10° C. Despite the shortcomings of the techniques used by previous observers, the more reliable data reported in this study (see Table IV) indicate that the reflex discharge is augmented in the cold environment.

Other possibilities for influencing the magnitude of reflex activity were examined. Although the data have not been presented in Results, adequate examination has revealed that there was no correlation of reflex magnitude with the time lapsed between cord isolation and start of hypoxic period (preparation time). However, preparation time never exceeded 180 minutes and was usually only 30-50 minutes long. There was no correlation with season, though the availability of frogs limited experiments to principally the summer and autumn months.

The capability for making integral measurements on frog reflex discharges in the spinal nerve was established only after overcoming great technical difficulties with isolation from noise. These difficulties were never surmounted for integration of microelectrode recordings. Therefore, amplitude measurements were made on focal potential fluctuations whose synchrony did not change. The focal presynaptic signal and the focal antidromic discharge did not change appreciably in duration during the experimental periods except when complete reflex failure was approached. The amplitude measures in the study have been used only to identify the presence or absence of a specific response, and only as a rough index of response magnitude. In summary, it has been concluded that the magnitude of reflex discharge of a population of spinal motor neurons can be measured in the spinal nerve. The integral measure is satisfactorily free from contamination by sources of electrical change other than axonal discharge and affords a more reliable estimate of the amount of reflex discharge than can be obtained by other methods.

Periods of Time

Since this study has been concerned with the temporal course of events, it is necessary to re-define and explain the criterion selected for the measurement of survival and preparation times.

The survival time constitutes the time from onset of the experimental period (hypoxia or ouabain) to the time at which reflex magnitude decays to one-half the magnitude of discharge during the control period. The selection of a relative measure of magnitude permitted normalization of the results. The selection of the time to 50% decay as a measure of the survival time, was necessitated by the slow non-linear decay of activity during hypoxia. The decay of hypoxic reflex activity was most rapid from the peak of augmentation to about 50% of the control levels. During the remainder of the hypoxic period the rate of decay diminished and the magnitude approached the zero axis very slowly. This slow approach to zero would have afforded a very insensitive measure of survival. Furthermore, in the experiments in which hypoxia was continued long past the criterion, with one exception, the reflex magnitude did not go to zero even after 3 hours of hypoxia. The 50% survival time, therefore, was a convenient measure of duration because, at this time, differences between experimental groups would most likely be greatest.

The preparation time was measured from the moment the dissected and isolated cord was placed in the experiment chamber to the moment the hypoxic episode was begun. During this period of time all spinal cords were uniformly exposed to Ringer's solution saturated with 95% O_2-5% CO_2. During the preparation period the spinal cord was positioned in the chamber and the necessary procedures were implemented for stimulating and recording from various sites of the cord. When the cord was finally ready for the experiment, a series of control records were made. The preparation time extends to the end of this control period at which time the experimental procedure (hypoxia) was begun.

It will be recalled that significant correlations were discovered between survival time and preparation time in two combinations of experimental groups. The combined observations of survival times of paired monosynaptic and polysynaptic reflexes in experiments which did not involve preparation for micro-electrode penetration (Group I) and experiments which did involve such preparation (Group II) was one instance of a significant correlation. The combined observations of survival times of discharge to dorsal root excitation at 4 and 40 second intervals was the other instance of this correlation. No group of experiments involving only a single variable exhibited a correlation between survival and preparation times.

One may well question the advisability of treating as one population the Group I and Group II experiments which were handled so differently. Obviously, correlation does not imply causation, and the observed correlation is further suspect since the survival times of the two groups are also correlated with manipulations necessary for micro-electrode insertion.

A proposed correlation of survival times with preparation time would be more meaningful if it could be observed within one experimental group or at least in a population established by combining two groups whose mean survival times and experimental manipulations were similar. It should be remembered that there was no significant difference between the survival times under hypoxia of cords stimulated at 4 and 40 second intervals (17.5-19.5° C). Since the null hypothesis cannot be rejected, the polysynaptic survival times of these two groups can be lumped together and considered as a single population, with more justification than can be mustered for the Group I-Group II combination. Though neither of the two groups separately showed any correlation, together there was a significant ($p < .02$) correlation of hypoxic survival times with preparation time. This observation on the polysynaptic reflex strengthens the probability that the correlation observed in the combined Group I and II population is not an artifact of the mechanics of preparation. In contrast to the population of Group I and II experiments, the composite population of polysynaptic survival times was obtained from cords not prepared for lateral column stimulation or micro-electrode recording. Therefore, there are two populations of experiments each handled in a different fashion, yet both exhibit a correlation of hypoxic survival time with preparation time.

The basis behind the correlation of hypoxic survival time and preparation time remains a mystery. It is possible that there is a true functional change occurring in the spinal cord during the period of isolation before hypoxia. The recovery from spinal shock has been considered as a factor, but the observations of reflex magnitude or amplitude do not reveal any consistent tendency to increase during the control period, as one would expect if spinal shock was wearing off. Furthermore, most investigators who have had experience with this preparation feel that spinal shock disappears within the first five minutes after spinal transsection[1]. However, there may be some more subtle change in the spinal cord associated with spinal shock that does not affect reflex magnitude but does influence the resistance of the spinal cord to hypoxia.

Another reasonable possibility to explain the significance of preparation time is that there was a depletion of metabolic reserves during the 10 minute dissection period in spite of the care to keep the cord bathed in oxygenated Ringer during dissection. If metabolic reserves are significantly depleted and if these reserves are only slowly replenished, then the resistance of reflexes to hypoxia might be expected to increase with longer preparation times.

It is important to point out again that variability in preparation time was too small in any one group to show a correlation with hypoxic survival time. Therefore, none of the conclusions drawn from these experiments need be concerned with variations in preparation time. The subject has been introduced into this Discussion only because it appears that it is a variable which should be considered in the design of similar experiments in the future. The correlation was quite unexpected; there is no indication

[1] Personal Communication with J. M. Brookhart, 1969.

from the literature that other investigators are aware of the possibilities implied by the above considerations.

IMPLICATIONS OF THE PATTERN OF HYPOXIC REFLEX FAILURE

Differences Between Polysynaptic and Monosynaptic Reflexes

The observations reported here provide for the first time information about the susceptibility of the monosynaptic reflex in the frog to hypoxia. This reflex has received little attention but offers the possibility for direct comparison of pure monosynaptic and polysynaptic reflex magnitudes and their pattern of hypoxic reflex failure in the frog spinal cord.

Both types of reflexes undergo augmentation in the magnitude of motor neuron discharge during the initial stages of hypoxia. The augmentation of polysynaptic discharges is greater than the augmentation of the monosynaptic response. The augmentation of the monosynaptic response is probably due to a recruitment of undischarged motor neurons since the duration of response is too short to provide for an increase in the frequency of discharge. On the other hand, the augmentation of polysynaptic activity could have been due to recruitment as well as to an increase in the motor neuron discharge frequency. The pattern of reflex failure, initial augmentation and subsequent decay, shows the same general sequence of events that Kirstein (44) observed in feline spinal reflexes. However, feline reflexes survive for only two to four minutes, whereas the frog spinal reflexes survive for 35-100 minutes under hypoxia. Therefore the pattern is developed much more slowly in the frog, and this enables one to test the spinal cord for reflexes at intervals long enough to avoid neurophysiologic interactions between the responses to succeeding stimuli. Kirstein's figures indicate that the monosynaptic reflex endured perhaps twice as long as the polysynaptic reflex, but he did not tabulate his data, nor give any indication of the reproducibility of his observations. It is interesting to note, therefore, that in the frog there appears to be some evidence to suggest that the monosynaptic reflex is slightly less susceptible to hypoxia than the polysynaptic reflex. However, the statistical significance of these observations ($p=0.06$) is only marginal for rejection of the null hypothesis and one cannot conclude with great confidence that there is a difference between the survival times of the two kinds of reflexes during hypoxia without further experimentation. If there is a difference it is small compared to those observed by Kirstein in the cat.

There may be a functional and/or structural basis for a difference in the susceptibility of polysynaptic and monosynaptic reflexes to hypoxia. Since the polysynaptic reflex travels a multi-neuronal path to the motor neuron there are probably a large number of loops and internuncial interconnections. The spread of afferent activity therefore has many possible routes to the motor neuron. One might expect that during hypoxia, in spite of the loss of a few of these elements, the reflex could be sustained by alternate pathways to the motor neuron pool. On the other hand, since the monosynaptic reflex involves a direct relation between primary afferent and motor neurons, no alternative pathways are available if any of the primary afferents fail and the reflex discharge must necessarily decrease. On this basis one might predict that the monosynaptic reflex would be more susceptible to hypoxia than the polysynaptic reflex. Van Harreveld made exactly this observation on cats when he compared naturally induced tendon reflexes with flexion reflexes. However, in the frog, the lateral column input is extremely powerful and probably each motor neuron receives terminals from several different lateral column fibers. If only a few of the axo-somatic terminals are necessary to discharge a motor neuron, synchronous activation of all lateral columns fibers may produce a surfeit of input (over-kill). In this case the hypoxic depolarization of part of the afferents might be without effect on the magnitude of reflex discharge. Therefore, when synchronous supramaximal stimulation of reflex afferents is used to produce reflex discharge, a somewhat different pattern of reflex failure could be expected than that observed when natural stimuli (e.g., tendon stretch) are used. If in the frog there is slightly more security in the monosynaptic pathway, it is probably because of its powerful axo-somatic input.

There is no reason to suspect that the metabolic disturbances caused by hypoxia are different in different elements of the two reflexes. Evidence has, however, been produced which indicates that all kinds of neurons are not uniformly susceptible to hypoxic depression (41,44,54,56,69). It has also been proposed that different subneuronal elements are differentially depressed during hypoxia. Differential susceptibility has been ascribed to differences in size of neurons or of subneuronal elements (41,56). On this basis, the smaller neurons are more readily damaged by hypoxic episodes. Histologic observations made on animals which have survived hypoxic episodes show internuncials to have been more severely damaged than motor neurons (45,53). However, subneuronal elements such as dendrites, and axon terminal branches are smaller than the cell body and in turn may represent better candidates for the weakest link in the reflex path than cell bodies (41). Since these subneuronal elements are common to both types of reflexes, there is no a priori reason to predict great differences in the survival times of polysynaptic and monosynaptic reflexes.

Differences in Presynaptic and Postsynaptic Susceptibility to Hypoxia

Attempts to pinpoint the site of hypoxic reflex failure were not entirely successful in these frog studies. What appears certain, however, is that hypoxic failure does not begin with failure of motor neuron excitability. The frog spinal motor neuron receives its monosynaptic input via axo-somatic synapses (146). Therefore, antidromic excitability is a true test of postsynaptic excitability. Since the monosynaptic synapses on the feline motor neuron are not confined to the soma (146), antidromic somatic excitability is not as reliable a test of postsynaptic excitability as in the frog. It is possible to imagine that hypoxia could cause depolarization of feline motor neuron dendrites causing postsynaptic failure to orthodromic input while the cell body remained excitable to antidromic invasion.

The Problem of Presynaptic Failure

Since the motor neuron soma in the frog retains its excitability after reflex failure has occurred, and since the peripheral afferent axons continue to conduct during hypoxia, it appears highly probable that the reflex failure occurs because of functional deficiencies related to the synaptic region. There are two separate processes in the synaptic region which might develop such functional deficiencies. The first is failure of afferent conduction to the terminals which, by default, would prevent synaptic transmission. The second is failure of the synaptic secretory or receptor mechanisms.

It is possible to observe signs of primary afferent activity with a micro-electrode placed in the motor neuron pool. The presynaptic signal is a synchronous rapid positive-negative fluctuation which is followed by a larger, more enduring postsynaptic potential. Classical interpretation of the components of the presynaptic signal ascribe the initial positivity to the approaching wave of activity distal to the recording field of the micro-electrode. The potential swings negative as the activity spreads into the recording field and invades the afferent terminal elements in the motor neuron pool. Brooks and Eccles (35) have suggested that the positive portion of the presynaptic focal signal (p wave) is due to activity in the parent axon and the negative portion (n wave) is due principally to activity in the terminal branches of the parent axon. On this basis, one would expect the positive portion to be unaffected by hypoxia since axons, at least in peripheral nerve, are quite resistant to hypoxia. If, as Eccles has hypothesized, failure of reflex activity during hypoxia is the result of depolarization of the terminal axon branches, then the negative wave would be expected to disappear and the positive component to increase with concomitant failure of synaptic transmission and postsynaptic response. This pattern of reflex failure was not observed in the frog. Both the p and n waves decreased in amplitude during hypoxia. Focal potential studies drawn from the literature (56) do not present convincing evidence that the p wave is more resistant to hypoxia than the n wave. One must either question Eccle's interpretation of what elements contribute potential to the p wave or conclude that the intraspinal axons of the lateral column are more susceptible to hypoxia than peripheral axons. The model proposed by Eccles (Figure 3) is an oversimplification of the complicated spatial arrangements of the intraspinal elements. There is not enough information at present to suggest any new interpretation of the presynaptic signals, but a decrease in amplitude of the presynaptic focal potential during hypoxia is consistent with the idea of presynaptic failure.

It is impossible with techniques available in this laboratory to determine whether the failure of central synaptic activation is due to blocked invasion of presynaptic terminals or to failure of the secretion-reception mechanisms which normally result from such invasion. Thus, the possibility remains that the reflex failure in these experiments was due to the second type of functional deficiency in the synaptic region. The susceptibility of the myoneuronal junction to hypoxia has been studied in lieu of a CNS synapse (154). This research also failed to distinguish between hypoxic failure of presynaptic invasion by action potential and failure of the secretory mechanism. It did establish, however, that repeated episodes of hypoxia could cause changes in the amplitude and frequency of miniature end-plate potentials. Therefore, synaptic secretory processes are, in fact, influenced by hypoxia under certain conditions in the myoneural junction. Whether or not this information can be extended to the CNS synapse remains to be demonstrated.

It is tentatively concluded, therefore, that the reflex failure in the frog spinal cord is presynaptic in nature and involves failure of presynaptic conduction. At present it is unknown as to whether conduction failure precedes or is concurrent with a possible failure of synaptic transmission mechanisms.

Metabolic Relationships to Hypoxic Reflex Failure

As mentioned previously, the feature that distinguishes the pattern of hypoxic failure in the frog from that in the cat is the length of the survival time. The frog spinal cord undoubtably has a lower metabolic rate at 18° C than the cat at 36° C. The survival time could be postulated to be a function of the length of time it takes for depletion of the metabolic reserves in the frog spinal cord. Obviously, any change in metabolic rate would result in a change in the time required for these reserves to be depleted. These reserves should be drawn upon more rapidly during activity than in the resting state. On this basis a hypothesis and its correlary were tested experimentally. If nervous discharge is a significant component of the total metabolic demand then it may be predicted that increases in reflex discharge would cause a decrease in hypoxic survival time by increasing the rate at which metabolic reserves are consumed. It would also follow that decreases in resting metabolic demand should cause an increase in hypoxic survival time. Simply stated, this hypothesis predicts an inverse relationship between survival time during hypoxia and metabolic rate. The experiments were designed to test this hypothesis by first changing the stimulus interval to effect a change in the metabolic drain due to discharge and secondly, to change the environmental temperature and thereby influence basal metabolic rate. Since an order of magnitude change in reflex discharge resulted in no change in survival time one must conclude that the energy cost of reflex activity in the frog spinal cord is considerably less than that required by basal metabolism. The inverse correlation of environmental temperature with reflex survival time under hypoxia confirms the prediction that survival time is indeed related to resting metabolic rate.

It should be pointed out in support of this conclusion that other more direct measures of metabolic activity have been applied fruitfully to peripheral nerve preparations. Such measures as phosphate production, oxygen consumption, and heat production, indicate that nervous activity does indeed consume energy (14, 107, 138,139). Peripheral axons have relatively small surface area to volume ratios compared to central neurons and the number of ions moved during each action potential (especially in myelinated nerve) is so small that concentration gradients are not greatly affecte [(14), page 71, (138)]. Therefore, high rates of stimulation and extremely sensitive metabolic measures are necessary to detect any increase in metabolic rate during activity. The increased metabolic rate during activity is thought to be the result of increased active ion pumping during recovery from the action potentials (118, 137,139).

The problems of interpretation of measures of metabolism of CNS preparations are exceedingly difficult. With peripheral nerve one can simply measure the oxygen or phosphate consumed and divide by the number of action potentials, or surface area to get an estimate of the energy cost per action potential. This cannot be done in the CNS, however. First of all the CNS contains whole neurons whose surface areas per unit weight have not been measured, though they are undoubtably much larger than axons (103). How does one estimate the total amount of membrane depolarized or the total numbers of neurons excited, especially with polysynaptic reflexes? In order to make this estimation the number of elements discharging, their frequency of discharge, total surface area, ionic permeabilities, sodium pump rates, and ion fluxes are parameters of energy drain to be measured. Obviously, the problems are too complex at present to expect direct measures of metabolic rate with neuronal activity to be made on CNS tissue with the quantitation possible with axon bundles. However, Ito and Oshima (66), succeeded in measuring sodium pump rates in spinal motor neurons in response to ionophoretic injected cations. Their calculated sodium efflux value (77 p mole/cm^2/sec.) is more than twice that observed in giant squid axon. In their opinion the sensitivity of the cat spinal function to hypoxia is based upon dependence on oxidative metabolism to drive these very busy active ion pumps. The ionophoretic injection of azide caused depolarization at the rate of 10 mv/min which is near the value observed by others for the depolarization of motor neurons under hypoxia (56,60,61). Furthermore, inhibition of the sodium pump by ouabain caused a 30-50% fall in oxygen consumption in the cerebral cortex (118) which also indicates that a great deal of resting oxidative metabolism is devoted to ion transport. Since only small numbers of ions move across the membrane during an action potential it is not surprising that the added metabolism of nervous discharge is insignificant relative to the resting metabolic rate. However, if hypoxia causes reflex failure by depolarization of presynaptic afferent terminals, then survival time of orthodromic reflex activity is really a measure of the metabolism of these, the weakest elements of the reflex path.

The great advantage of the spinal cord over cerebral cortex is that input can be controlled, and output measured. Spinal cord reflexes provide a measure of function to relate to metabolic activity. The isolated frog spinal cord has the advantages of isolation from systemic influences and capability for better control and measurement of nervous activity. Thus, these studies present the base line data which will permit systematic use of the preparation in future experiments designed to relate function to metabolic factors. The author intends to exploit this preparation further in an attempt to gain a better understanding of CNS function and neuronal cell physiology.

THE MECHANISMS OF HYPOXIC REFLEX FAILURE

Hypoxia has been used in this research to produce a "metabolic disturbance". One might ask, what kind of disturbance, and how does this metabolic disturbance influence reflex activity? Current hypotheses have been identified throughout this thesis. Briefly, the concepts are as follows: Hypoxia results in depletion of energy reserves which are necessary to drive the active ion transport processes. Passive ion movement, uncompensated by active redistribution, would result in depolarization of the neuron membrane. As a first approximation it has been assumed that regions of the neuron that have the highest surface area to volume ratio should depolarize first. If these ideas about the mechanisms of hypoxic reflex failure are true, then simple inhibition of the sodium pump should result in the same pattern of reflex failure observed during hypoxia. It is possible to approach this problem pharmacologically.

Ouabain is considered by some to be a specific inhibitor of the sodium pump in certain tissues [(113), page 305]. Evidence was presented that the oxygen consumption of the frog spinal cord falls in response to ouabain poisoning in a fashion similar to that observed for the cerebral cortex. Preliminary evidence also indicated that the frog spinal cord contains a Na^+, K^+ activated ATP'ase, inhibited by ouabain. These results suggest that the action of ouabain on the frog spinal cord shows characteristics similar to those seen in tissues where ouabain is known to inhibit active sodium efflux. Therefore, it is assumed herein that the frog spinal cord is another of the many tissues whose active ion transport is inhibited by ouabain.

Apparently, no experiments have been performed to test the action of ouabain on spinal reflexes. However, it is worthwhile to predict what these effects might be, assuming ouabain acts to specifically inhibit active ion transport in central nervous system tissue. First of all, ouabain should cause reflex failure. The delay between onset of drug administration and reflex failure should be in part due to diffusion to the sites on the neuron membrane for active transport. There should also be a delay between the time that active transport is halted and the time depolarization has occurred in enough elements in the reflex arc to cause reflex activity to decrease. The rate of depolarization should be proportional to the rate of change of intracellular concentration which would, in turn, be influenced by membrane permeability and the ratio of surface area to volume. Thus, ouabain could be predicted to cause reflex failure in the same manner that hypoxia has been postulated to act. On this basis it appears that ouabain and hypoxia would cause reflex failure with similar patterns.

There are, however, some important additional facts to be considered before accepting the assumption that hypoxia and ouabain will cause similar patterns of dysfunction. Hypoxia primarily causes a depletion of energy reserves; secondarily, the depletion results in inhibition of active transport. The delay between the start of hypoxia and reflex failure will be due in part to diffusion of the stored oxygen out of the tissue. However, even when the neuron is hypoxic it should not begin to depolarize until its energy reserves are depleted to the point where they are rate-limiting for the operation of the sodium pump. The rate at which energy reserves are depleted depends upon the metabolic rate of anaerobic energy production. Only when energy stores are depleted to a level where the sodium pump activity is depressed, will the cell depolarize at a rate proportional to the ionic permeability and the surface area to volume ratio of the cell. It follows then, that while the rate of hypoxic reflex failure is dependent upon both the metabolic rate and the structural characteristics of the cell, the rate of ouabain induced reflex failure would be independent of neuronal metabolic rate and a function only of neuron structure.

Most investigators apparently do not consider the metabolic aspects of different elements of the reflex path when they discuss patterns and mechanisms of reflex failure. Both Eccles and Van Harreveld tacitly assume that the rate of metabolic depletion in the elements of the reflex path is an unimportant factor, because they consider hypoxic reflex failure solely on structural (surface area to volume) grounds. There is evidence that the metabolic rate is different for the various elements in the reflex path. For example, Lowry (see Tower (183), page 1802) has suggested that metabolic activity of grey matter is centered principally in the dendrites. Since the soma is the site of protein synthesis, one would expect a higher metabolic rate in this structure in comparison to the axon, whose principle metabolic requirement seems to be the maintenance of the sodium pump. Nevertheless, it would be difficult to predict with certainty which element in the reflex path would fail first under hypoxia because there is not enough detailed information about the relative metabolic rates or potential for anaerobic metabolism in the various elements of the reflex path. If it can be assumed that ouabain has indeed a direct effect solely on pump activity, one could predict which elements of the reflex path would be most sensitive to ouabain since much is known about their structure and relative surface area to volume ratios. Should a comparison of the patterns of hypoxic reflex failure and ouabain reflex failure reveal obvious differences, these differences might be related to the relative metabolic rates of the elements undergoing depolarization. The problem then becomes one of kinetics.

From the hypothesized mode of action of ouabain, it can be predicted that ouabain should cause dendrites and terminal axons to depolarize before the cell bodies and parent afferent axons which give rise to presynaptic terminal branches. Therefore, depolarization of presynaptic terminals should occur first and cause loss of the presynaptic focal potentials, and orthodromic motor neuron discharge before failure of the antidromic response.

The results from the frog experiments were not as predicted. Indeed there was reflex failure, but the pattern of failure was not at all similar to that seen during hypoxia. In the presence of ouabain, failure of reflex activity was not characterized by the relatively slow simple decay of reflex discharge seen during hypoxia,but instead failure was accompanied by several abrupt fluctuations in magnitude (see Figure 27). The results of most experiments exhibited at least one episode of transient fluctuation in reflex magnitude. Most of these episodes were characterized by an initial abrupt, and sometimes, complete loss of reflex excitability which was followed by a temporary "rebound" or recovery of response to stimulation. In three experiments there were two episodes of decline in reflex magnitude but the level of recovery following the second episode was less than in the first.

The conclusion drawn from the observations of focal potential records is that reflex failure is the result of inexcitability in all of the elements of the reflex path. Antidromic focal potential disappeared even during the initial temporary loss of reflex excitability. The pattern of changes was so rapid that observations made at 40 second intervals were unable to provide, with certainty, information as to which element in the reflex path became completely inexcitable first. There were four experiments where both phases of the presynaptic focal potentials were present after the negative phase of the antidromic focal potential disappeared, which might indicate that the motor neuron became inexcitable earlier than the lateral column fibers.

There is some question, however, as to the mechanism underlying transient inexcitability of the reflex elements. Since the disappearance of the reflex and antidromic discharges was only temporary, and since the recovery was quite rapid, the motor neurons could not have been made inexcitable by passive ion equilibration. It would seem more likely that ouabain in some manner caused a temporary imbalance between inhibitory and excitatory activity which was so extreme that reflex excitability was temporarily overcome. If unpredictable functional changes occur in the spinal cord during ouabain poisoning, it would be difficult to test the hypothesis concerning its mode of action by using gross recording electrodes. Uncontrollable changes in the balance of excitatory and inhibitory input to motor neurons, perhaps by spontaneous internuncial activity, would invalidate the present experimental design as a means for testing the structural hypothesis for susceptibility to metabolic interference. Intracellular recording from motor neurons would permit a more rigorous evaluation of the nature of spontaneous input to the motor neuron and a differentiation between hyperpolarization (from IPSP's) and depolarization as the cause of motor neuronal inexcitability. These experiments have not yet been performed, but it is hoped that in the near future a more detailed analysis of the effect of ouabain on spinal reflexes can be accomplished.

In summary, the ouabain experiments failed to yield information about the susceptibility of different elements to passive ion fluxes. The spinal cord responses were probably complicated by neurophysiologic changes which could not be monitored by this experimental design. The studies performed with hypoxia involve consideration of metabolic as well as structural factors in determining the susceptibility of reflex elements to hypoxia. This susceptibility of function to hypoxia is based upon metabolic demands present in the resting unstimulated cord and only secondarily on the level of activity. To what extent these metabolic demands are coupled to active transport is unknown, but it seems that the motor neuron is structurally and metabolically favored to resist the disturbances caused by hypoxia to a greater degree than any other element in the reflex path.

SUMMARY AND CONCLUSIONS

Experiments designed to influence metabolism while monitoring function were performed on the isolated frog spinal cord. Quantitative estimates of motor neuron discharge, obtained by electronic integration of spinal nerve potentials, provided a means for determining the effects of metabolic alterations on nervous discharge. A large number of experiments were carried out to establish the pattern of monosynaptic and polysynaptic reflex failure during an episode of hypoxia. The results of these experiments differ from the results obtained by others from cat spinal cord in the following manner:

1. Hypoxic reflex survival time is much longer in the frog.
2. Monosynaptic reflexes are not markedly more resistant to hypoxia than polysynaptic reflexes.
3. The excitability of the motor neuron soma and axon is much less susceptible to hypoxia than other elements of the reflex arc.

Several experiments were performed to determine the site of reflex failure. Interpretation of the focal potential records indicated that the monosynaptic afferent axon and its terminals were the first elements in the monosynaptic reflex path to fail. It was concluded, however, that this presynaptic failure may or may not occur <u>initially</u> in the fine terminal branches.

The apparent failure of the lateral column axon indicated by the focal potential studies is inconsistent with evidence presented that axons are extremely resistant to hypoxia, and demands a reevaluation of the basis for the interpretation of the focal signs of presynaptic impulse arrival. The presynaptic focal potential may be complicated by activity not directly associated with the motor nucleus in which the micro-electrode resides. Furthermore, focal potential signals cannot be used to distinguish between conduction failure in the presynaptic axons and failure of synaptic secretory mechanisms. Consequently, it was concluded that the genesis of the presynaptic focal potential is not sufficiently well understood to permit identification of the site of reflex failure.

The mechanism of hypoxic reflex failure undoubtedly has a metabolic basis. It was hypothesized that the rate of reflex failure would be directly proportional to the metabolic rate (the survival time would be inversely proportional). Changes in the basal metabolic rate were assumed to occur when the environmental temperature was changed. Since hypoxic reflex failure was more rapid at warmer temperatures than at colder temperatures the dependence of function on oxidative metabolism is considered proven.

The increase in nervous activity produced by increasing the stimulus rate 10 times did not increase the metabolic rate over basal conditions enough to alter survival time. It must be concluded that the energy cost of nervous activity is relatively small compared to that required simply for maintenance of excitability.

The mechanism of hypoxic reflex failure was proposed to consist of failure of active ion transport and consequent depolarization of neuronal elements. It has been suggested that the rate of depolarization would be a function of relationship of the surface area to the volume of the neuronal element. This suggestion is based on the implicit assumption that active ion transport is completely inhibited in all elements at or near the same time. It was concluded from the research reported herein that the vulnerability of various neuronal elements to hypoxia cannot be solely ascribed to surface area-volume considerations. Ouabain, considered to be a specific inhibitor of active cation transport, resulted in a pattern of reflex failure quite unlike that associated with hypoxia. Reflex failure was rapid and oscillatory, and failure of conduction occurred in both pre and postsynaptic structures at about the same time. It is suggested that the vulnerability to the effects of hypoxia, although ultimately produced by ion transport failure, would be conditioned by the availability of metabolic energy reserves. These metabolic reserves should be different in the various portions of the neuron.

The frog spinal cord is an excellent preparation which has many advantages over the _in situ_ mammalian preparations. By virtue of the above research future experiments with this preparation can be performed to investigate the intriguing problems of metabolic correlates to nervous activity.

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APPENDIX

Operational Description of Figure 29

The camera shutter relay is activated when the transistor is biased on. This condition prevails when the input to the transistor is at least 1 volt positive. Two gates in the Figure were effectively wired as a single three input gate. When all inputs to the gate are low the output is positive 3 volts, and this is sufficient to trigger the transistor and shutter. One gate was used simply as an inverter. The flip-flops toggle on the negative going portion of the input pulse. Between pulses the output of the inverter is high which causes a low ouput from the gate. The input pulse will cause the output of the inverter to swing low. This in turn causes the output of the gate to swing high to trigger the shutter only if the other two inputs are low as would be the case initially. At the end of the first input pulse the first flip-flop will toggle high. The operation is the same for the next three input pulses only. The four flip-flops are in reality arranged as a four bit binary counter. Therefore a picture of the stimulus response is taken for the first four of each set of 16 stimuli or for 25% of the responses.

FIGURE 29. Timing Circuit for Experiments where Stimulus Interval was 4 Sec.

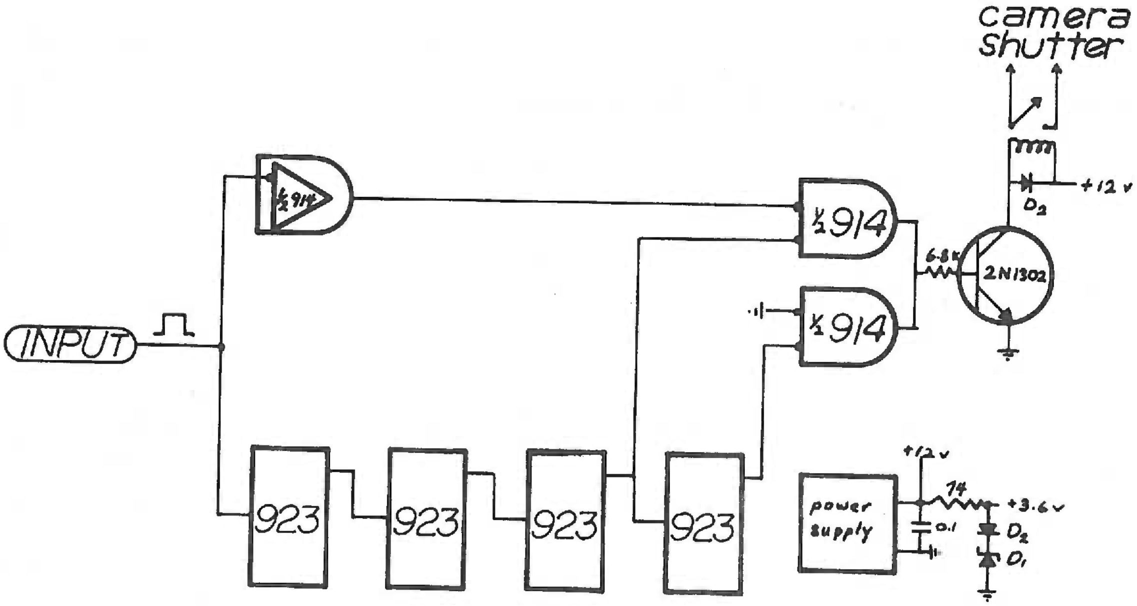
Components:

914 - Fairchild micrologic dual NAND gate
923 - Fairchild micrologic RS Flip-flop
D_1 - Germanium signal diodes
D_2 - Zener 3.0 volt diode
Resistances in ohms
Capacitances in microfarads

Truth table for the NAND gate

Input voltage	Output voltage
Low-Low	High
Low-High	Low
High-Low	Low
High-High	Low

(See Appendix for a functional description of this Figure.)


camera
shutter
+12 v
D2
½ 914
½ 914
½ 914
6.8k
2N1302
·1
INPUT
923
923
923
923
power supply
+12 v
74
+3.6 v
0.1
D2
D1

COMPOSITION AND PREPARATION OF FROG RINGER

Composition and Stock Solutions

Compound	Ringer Concentration g/l	mm/l	Stock Sol. g/l	Stock/Ringer ml/l
NaCl	4.28	73.25	171.2	25
$NaHCO_3$	2.94	35.00	58.8	50
KCl	0.23	3.13	9.2	25
Na_2HPO_4	0.25	1.76	10.0	25
$NaH_2PO_4 \cdot H_2O$	0.08	0.59	3.2	25
Glucose	2.92	16.18	116.8	25
$CaCl_2 \cdot 2H_2O$	0.22	1.99	8.9	25

Preparation

1. Add appropriate volumes of stock solution (except $CaCl_2$). Dilute to the required volume less the volume required for the $CaCl_2$.

2. Gas solution with a gas mixture 5% CO_2 for 15 minutes. Add $CaCl_2$. The pH should be 7.4.

Note: In the experiments herein, 5 liters were made up at one time without any glucose and stored in the cold room. Before the start of the experiment 2.9 grams of glucose was disolved in one liter of the Ringer and the solution gassed with the appropriate mixture.