A STUDY OF GLUCOSE UPTAKE BY FROG SPINAL CORDS DURING REST AND EXCITATION

by

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INTRODUCTION

INTRODUCTION

Early attempts to unravel the complexities of neural metabolism and function were hindered by a multitude of factors. Work had to be done with the basic organic chemistry of mixtures and substances, much of which is only partially understood even with modern methods. Only recently have enzyme studies revealed information about the nature of the glycolytic and oxidative aspects of metabolism. The development of micromethods of enzyme analysis by Lowry in St. Louis, where the studies in micromethods of the Copenhagen school led by Linderstrøm-Lang are being applied to the nervous system, has led to a major break in the binding chains of the field. A second blocking factor is found in the fundamental difficulty related to the nonhomogenous nature of the nervous system, both in organization and function. The success of histology in delineating the complex microstructure of nervous tissue has emphasized this. The main functional unit of the nervous system is the neuron. This structure lies embedded in a syncytium of neuroglial elements, the biochemical and functional role of which remains obscure. Therefore, any attempt to relate the mechanism of excitation and conduction to metabolism depends on the separation of the chemistry of the functional elements from the structural components of the tissue. Yet, to disregard the metabolism of the neuroglial cells is not justified. It is improbable that their role is a "passive" one. Nageotte suggested that the function of the neuroglia was a nutritive one as they appeared to contain secretory

granules (44). Electronmicroscopy has revealed the existence of granules within neuroglia which are occasionally found in large clusters as if they were in the process of being discharged into the intracellular space (52).

One question that has arisen from the results of work done with the knowledge and refinements of modern methods is that of the relations between metabolism and function. To what extent does metabolism only maintain structure, and to what extent does it contribute to the working function of the tissue?

Relationship of chemistry to function.

According to Bernstein's ionic or membrane theory of bioelectric activity, the internal negativity of muscle and nerve cells is accounted for by semi-permeable characteristics of cell membranes which result in the unequal distribution of ions between the interior of the cell and its external environment. The interior is some 10 to 100 times richer in K^+ than the extracellular fluid, while the concentration of Na^+ internally is only about one-tenth of that externally. Such inequalities probably arise and are maintained from metabolically driven ion pumps which extrude Na^+ and take in K^+. The anatomic site of these pumps is most likely the cell membrane, but the nature of their mechanism of action remains undetermined. In order to transport ions against such concentration gradients, the cell must expend considerable energy. As a result of the maintenance of such concentration gradients, the cell membrane is electrically polarised. The resting membrane potential can

be accounted for by relations between internal and external ion concentrations (30). Membrane permeability changes elicited in response to stimuli allow the ions to redistribute themselves along their concentration gradients. The membrane potential correspondingly varies with the influx and/or efflux of the ionic charges. Thus, the concept has arisen that the depolarisation of conductive tissue is metabolically passive, while maintenance of resting membrane potentials and repolarization are processes requiring energy. There is considerable evidence that respiratory activity increases during excitation (19, 20), and the increased production of heat during nerve conduction was discovered only a quarter of a century ago (28). The increase in respiratory activity observed with stimulation is not accompanied by increased phosphorylation, so that an uncoupling of oxidation and phosphorylation may be taking place (51). Evidence from many sources indicates that excitation is accompanied by a decrease metabolic energy output. This has been observed in brain slices (40, 41), brain mitochondria (4), and sartorius muscle (7, 35). The orthophosphate - P^{32} uptake by frog nerves is inhibited during excitation (5). Electrical stimulation as well as dinitrophenol results in a decrease of the K^{42} turnover of rat brain and muscle mitochondria, and in a considerable decrease in the K/Na ratio (2). The concept that K-Na transport and phosphorylation are closely related is further supported by the observation that depletions of intramitochondrial potassium result in decreased phosphorylation (5, 31).

Exogenous substrates of the neurone.

Glucose is the only substance which is taken up by the brain in

large enough quantities from the blood to satisfy its energy requirements (29). Himwich also postulates that the brain does not contain a large enough pool of substrates to maintain function in the face of a loss of a constant uninterrupted renewal of such substances by the circulation. The dependence of the brain on a minute-to-minute supply of glucose from blood for maintenance of function and electrical activity has become an axion of neurophysiology (62). There is little doubt that glucose is the chief substrate of the central nervous system during rest and activity (6). Strong evidence is available indicating that the brain derives its energy for normal function almost exclusively from the oxidation of glucose (55).

Statements of the above nature are readily found in both the past and current literature dealing with neural metabolism. The experimental work upon which such statements are based is impressive in amount and quality.

Sokoloff has briefly summarized what he terms "evidence for the obligatory aerobic utilization of glucose by the central nervous system" mainly from three groups of *in vivo* observations (55).

1) Impairment of CNS activity produced by glucose deprivation. Such observations are related to well known changes in the mental state of man resulting from hypoglycemic states, and the correlation of the degree of behavorial and functional impairment with the degree of hypoglycemia.
2) Recovery from the effects of hypoglycemia produced by glucose administration. In man, the symptoms of insulin coma can usually be reversed in minutes by glucose administration.

3) The relative inability of other substrates to produce recovery from effects of hypoglycemia.

Three major methods are available for determining whether a substance is utilized as a metabolic substrate (56). The first being the ability of the substance to support metabolism. This has been shown to be true in CNS tissue of various experimental animals and man for the hexose sugars; glucose, mannose, fructose, and to some extent galactose (56). Certain non-CHO substances capable of sustaining cerebral oxygen uptake include derivative of the glycolytic and tricarboxylic acid cycles and glutamate. It must be kept in mind that although a substance exhibits the ability to sustain tissue respiration when present in an incubation medium, this is no indication of the role it plays in the intact organism. The majority of types of procedures used in these studies have consisted of work with bries, homogenates, tissue slices, and other preparations which destroy tissue integrity and cell structure. Results obtained from these preparations reveal only information about tissue potential and are not true reflections of rate and function in the intact organism. Such is exemplified by evidence for the usage of glutamic acid as a cerebral metabolite when the inability of glutamic acid to pass the blood brain barrier has been observed in rats and mice (54). This type of contradictory evidence has also been seen for a number of members of the tricarboxylic and glycolytic cycles (15). Therefore, experiments conducted with preparations in which organ and cell structure are interrupted do indicate tissue metabolic potential

but do not differentiate between the exogenous or endogenous supply of the metabolite in the intact organism.

A second method is that of measurement of RQ values. The measured RQ of excised brain in the presence of glucose approaches unity (16, 17). The respiratory rate of the monkey brain _in vivo_ is unity (53). By the nitrous oxide method, Kety and Schmidt have demonstrated similar results in man (37).

The third method consists of the quantitative comparison of oxygen uptake and the absorption of the foodstuff. In excised brain, the oxygen uptake seems to correspond to the oxidation of glucose, and A-V differences in oxygen uptake and glucose utilization indicate the same conclusion _in vivo_. One must accept studies based on A-V differences with care. Arterial samples can be obtained from any artery. A problem arises with venous samples from cerebral tissue as one obtains a mixed venous drainage with the sample being contaminated by blood from extra-cerebral tissue. In man, a fairly representative sample can be obtained from the superior bulb of the internal jugular vein (43). Such determinations are not meaningful unless correlated with blood flow rates to yield quantitative information on uptake rates. These studies allow the comparison of the utilization of various compounds such as glucose and oxgyen with the production of carbon dioxide and lactate or pyruvate. However, to measure rates of flow concomitantly with A-V concentration differences necessitates such extensive operative manipulations that the procedure becomes one of an _in vitro_ study _in situ_ rather than one of an _in vivo_ nature.

Failure to demonstrate significant A-V differences does not preclude a substrate from a role in cerebral metabolism. The fact that stores of glucose and glycogen in CNS tissue are low is indicative of rapid utilization. Also, the factor of the rapid blood flow through the brain produces A-V differences of small magnitudes which may be difficult to detect (55).

Nerve appears to contain the enzyme systems involved in glycolysis and oxidation via the Krebs' cycle. Therefore, with the combination of a cerebral respiratory quotient approximating one, an almost stoichiometric relationship between the glucose and oxygen uptake necessary for the complete oxidation of the glucose, the presence of enzyme systems necessary for the metabolism of glucose, and the absence of a significant arterio-venous difference for any other energy rich compound, strong supporting evidence for the concept that energy for cerebral metabolism is derived almost exclusively from the oxidation of glucose is available.

The results of Geiger's perfusion experiments with an intact cat brain indicate that glucose transfer into brain may be dependent upon the presence of certain substances in blood. In a series of experiments in which a cat's brain _in situ_ was perfused with simplified blood[1] by means of the isolated cerebral circulation, it was found that the transfer of glucose from blood to brain was interrupted and the brain preparation could not be kept alive for periods longer than 60-90 min. During

[1]The simplified blood perfusate consisted of washed red blood cells suspended in a saline solution containing 10% bovine serum albumin. The final volume of the suspension was adjusted with Ringers to contain 35-40% RBC and 7% bovine serum albumin. To this was added glucose. Therefore, except for glucose, the simplified blood perfusate was devoid of organic substances normally present in blood (24).

this period, lactic acid concentrations in the brain steadily increased while glucose concentrations decreased despite high concentrations in the perfusate. This coincided with a decrease in the electrical signs of brain activity. Fresh liver introduced into the perfusion cycle kept the brain alive for periods of up to four hours without noted disturbances in CHO metabolism. Similar results were observed with the addition of liver extract or 30-40 ml of fresh blood drawn from the femoral artery of the cat being perfused to the perfusing solution. No restorative effect was obtained with the vit B's, ATP, adenylic acid, DPN, TPN, creatine PO_4, insulin, coenzyme A or ascorbic acid (24). It was therefore concluded that disturbances in brain function result, at least in part, from a lack of substances which are normally contributed by blood. Further investigation of the components of the liver extract led to the finding that the active constituents were the pyrimidine nucleosides, cytidine, and uridine. In the absence of these, the brain developed impermeability to glucose and a depletion of galactoside (restored by uridine) and phospholipid (restored by cytidine). Both nucleosides were required to restore normal CHO metabolism (25). During glucose free perfusion, the CHO reserves of the brain are used up rapidly; the glucose metabolizing enzyme systems and possibly the transfer mechanisms are damaged. Such systems require reactivation in order to resume normal functions. These inferences have been derived from the observation that, following glucose free perfusion, glucose added to the perfusate was not utilized by the brain unless the simplified blood was

fortified by the addition of liver substances (3).

Further evidence for the chemical alteration of blood glucose before its uptake and utilization by the brain is given by the observation of Geiger (23) that glucosamine is taken up and phosphorylated in the living brain at a time when the brain is unable to utilize glucose from the blood.

Another series of observations which stress the possible importance of intact membranes and chemical processes occurring in the membranes to the transport of substrates from the circulation to the neurone has been presented by Rafaelsen (45-49). It has been generally accepted that the major, if not the only, effect of insulin on central nervous system tissue is one of an indirect nature (i.e. nervous system manifestations of the hypoglycemic state resulting from the peripheral action of insulin). Himwich and co-workers demonstrated that brain slices from depancreatized cats were able to oxidize glucose (10). From this work, it was concluded that insulin was not required for the metabolic mechanisms of glucose oxidation in brain tissue under the experimental conditions. Rafaelsen, however, feels that this has been consistantly misinterpreted as "is not influenced by insulin" (45). It has also been accepted that insulin does not cross the blood-brain barrier. With such results recorded in the literature, the work dealing with insulin and the CNS has been sparse. Rafaelsen hasshown what he has termed a direct effect of insulin on CHO metabolism in the rat spinal cord (47, 48, 49), rat brain slices and isolated rat cerebellum (45, 46). He has shown significant differences in the glucose uptake rates, in the presence of insulin, between first and second brain slices of rat cerebral tissue. From this observation,

it was suggested that the effect of insulin is intimately connected with the structure of tissues. First brain slices from the convex side of the hemisphere have one side intact and are covered with pia-arachnoid, whereas the remainder of the slices have two cut surfaces. In his experiments, no effect of insulin on glucose uptake was seen in second and third slices or in cerebral homogenates, but a 10% increase was noted with first brain slices incubated in the presence of insulin in concentrations of 10^{-1} units/ml. It was from these observations that he postulated the necessity of intact membranes and tissue integrity for the action of insulin to be elicited.

Until recently, attempts to demonstrate fatty acid oxidation in neural tissues have been largely unsuccessful. Of numerous short-chain fatty acids tested, only beta-hydroxybutyrate appears to be oxidized by brain or nerve homogenates and mitochondria (4). During glucose free perfusion of cat brain, considerable amounts of phospholipids were found to disappear from the cerebral cortex, and it was not possible to account for them on the basis of lipids leaving the brain (3).

Endogenous Substrates.

Most of the important substrates and metabolites of the brain are present in trace amounts only (61). The amounts indicated below are per gram of whole brain.

1) Oxygen:

Cerebral tissue	90 µmole/gm
Cerebral vasculature	225 µmole/gm

This amount at a normal rate of utilization by human brain would

last only about 10 seconds.

2) Glycogen: 5 µmole/gm

3) Glucose: 4 µmole/gm

Therefore, the brain supposedly contains no significant store of "essential nutrients", but depends upon a continual supply from the cerebral circulation.

Evidence for the cerebral metabolism of endogenous substrates became prominent with the results of Geiger's perfusion experiments. Without liver inclusion in the perfusing circuit, he could account for the entire disappearance of glucose from the blood by lactic acid production. Yet there remained a persistent high O_2 consumption. This was taken to indicate oxidation of non-CHO compounds in brain (22, 24).

The fact that glucose is the only substrate taken up from blood into the brain in large enough quantities to sustain its energy requirements suggests that glucose is the carbon source for all main metabolic processes in the brain including production, by exchange or synthesis, of most amino acids, proteins, lipids, etc. In a group of experiments, Geiger and co-workers added uniformly labeled C^{14} glucose to their perfusing blood and noted the appearance of C^{14} in the respired CO_2 within a minute (27). The concentration rose steadily until it attained a constant level in approximately 30 min. and then remained steady for the duration of the experiment (30-60 min.). The phase of rising CO_2 concentration was taken to indicate the exchange of pre-existing glucose and its intermediates with labeled glucose supplied from blood. It was assumed that at the end

of this phase exchange with direct metabolites and other quickly exchangeable compounds had been completed. The second phase reflected the equilibrium of cold carbon and C^{14} from the perfused glucose. The ratio was considered to indicate the proportion of glucose and non-glucose compounds participating in respiratory CO_2 production. In all experiments with Metrasol convulsions, the O_2 consumption and total CO_2 production increased. No increase in the C^{14} output of the respired CO_2 was noted. Decreases were observed in some cases. The ratio between C and C^{14} indicated that much less carbon was derived from glucose than in the resting state. The extra O_2 consumed during convulsions was accounted for by oxidation of non-glucose or non-CHO substances (8, 24). Under resting conditions, C^{14} concentrations in respired CO_2 increased to concentrations of 29-32% and then remained constant.

Any discussion of the use of endogenous substrates in CNS metabolism requires simultaneous consideration of the differences between resting neural metabolism and metabolism during activation. In later work, Geiger concluded that endogenous substrate metabolism predominated during activity (21). This preponderance of non-CHO oxidation was observed even when glucose was present in the perfusing blood in normal concentrations (22). In a series of perfusion experiments involving Metrazol induced convulsions, it was found that during the first minutes of the convulsive period, glucose breakdown was accelerated while oxygen consumption increased two to three times. At the same time, lactic acid was formed at a rate roughly equal to the glucose uptake. As lactic acid accounted

for almost all the glucose which disappeared from the brain and blood during the convulsion, other non-CHO sources had to account for the increased O_2 uptake. No significant glycogen breakdown was detected. Carbon dioxide production remained at preconvulsive or lower rates. Therefore, glucose oxidation did not increase. After O_2 consumption returned to resting rates following the convulsions, glucose uptake from the blood gradually increased for about 20 min. while $C^{14}O_2$ output remained stable. Two to three times as much glucose was taken from blood in this period as could be accounted for by O_2 consumption and lactate formation. At the same time C^{14} labeled brain proteins and lipids increased (21). Such findings indicated the breakdown of non-CHO substances during activity and their resynthesis from glucose during a recovery period. Geiger also concluded that quantitatively, next to carbohydrate, endogenous proteins seemed to be the most important substrates of brain metabolism.

Geiger carried this work further. Knowing that glucose added to the perfusion blood was rapidly incorporated into free amino acids of brain and at slower rates into its structural components, his group determined the effects of convulsive activity induced by Metrazol or electrical stimulation on incorporation rates of C^{14} into proteins as compared to rates at rest (26). Resting experiments indicated incorporation rates which were slowly but progressively slowing down after 30 minutes of perfusion, and by comparison with rates in normal, awake, non-perfused cats rather higher. During convulsions incorporation rates increased. Rates were almost doubled by 10 min. stimulation periods following 30 min. of perfusion with C^{14}. For about 15 min. after the convulsive period, the

rate came to a standstill and even losses of radioactive protein were noted in spite of the continued presence of C^{14} glucose in the blood. Following this lag period, the incorporation rates again greatly increased. Forty minutes after the convulsions, two to four times as much C^{14} was incorporated into the post convulsive brain as into a resting brain during a comparable period of time. This work confirmed the fact that C atoms derived from glucose were incorporated into cerebral proteins. It also indicated three stages of effects following convulsive activity.

1) Period of stimulation: Here incorporation rates increased. This period also coincides with the breakdown and oxidation of non-CHO material in brain cells witnessed previously during convulsive activity. Resynthesis during refractory periods and asynchrony of neuronal activity during convulsions were possible explanations given to account for the increased incorporation.
2) Lag phase of incorporation: The authors found it difficult to understand why C^{14} incorporation should cease during this phase of reduced physiologic activity. It was considered possible that a soluble pool of metabolites present at the beginning of convulsions was rapidly used up by high rate oxidative processes and glycolysis. During this period, the rate of glucose uptake was considerably reduced. Thus, at the end of the period of convulsions lasting 10-15 min., the soluble pool of metabolites was exhausted and the C^{14} diluted by the influx of cold carbon from the breakdown of lipids and

other tissue components. The temporary lack of specific metabolites could have accounted for the absence of protein synthesis. This explanation is not entirely substantiated by the findings of considerable stability in the amino acid pool under varying conditions of activity observed by these same workers (11). It was therefore necessary to assume that the amino acids did not represent the only carbon pool which had the ability to replenish glycolytic and tricarbo-xylic acid cycle intermediates. The data appeared to be best understood by assuming that a source of cold carbon was feeding into and forming these intermediates at a rate which was at least equivalent to the rate at which they were formed from exogenous glucose taken up by the brain. The major portion of the radioactivity taken up as glucose which did not appear in the respiratory CO_2 was present in the acid soluble fraction which probably includes the glycolytic and cycle intermediates. This explanation again emphasizes the possible importance of the metabolism of both amino acids and other endogenous substrates.

3) Post convulsive state: In this phase, incorporation rates were greatly increased. The duration of the phase extended past the limit of experiments which was set at 90 min.

Compared with resting rates, the high incorporation rates associated with convulsions and the post convulsive state suggest a high turnover rate of protein with activity metabolism in brain. This conclusion is

supported by the finding of an increase breakdown of nitrogen containing substances and the prevalent oxidation of non-CHO substrates by brain during convulsions.

Brain does contain relatively high amounts of amino acids, both free and combined. These account for about 40% of the dry weight of the tissue (42). Seventy percent of the amino nitrogen fraction is composed of glutamic and aspartic acid and their derivatives (9, 58-61). This group, especially glutamic acid and glutamine, is present in brain in higher concentrations than in any other organ and is associated with metabolic systems specific to neural tissue (58, 60). Glutamic acid is utilized readily by the mitochondria of the brain (1, 13) and contributes to more efficient phosphorylation (i.e. higher P/O ratios) than does pyruvate (4). This amino acid comprises an intimate link between protein and carbohydrate metabolism and its possible metabolic role deserves consideration and exploration.

Further evidence indicating the importance of amino acids in CNS tissue metabolism is becoming prominent and impressive.

It has been observed that 41% of labeled glucose injected into the tail veins of rats was incorporated into the amino acid fraction of the brain in two minutes and a 75% incorporation was reached in 30 min (63). A general pattern for the appearance of radioactivity into the acid soluble amino acid component of brain has been observed by Geiger and his group (11). It did not seem to vary in any characteristic manner with either Nembutal narcosis or Metrazol convulsions. The results of

this group's work indicated that the first samples showed appreciable radio-activity in the combined amino acid fraction (5-10% of the specific activity of the perfused glucose). The second samples showed further increases, but not pronounced. In third samples, the activity found in the amino acid fraction was increased up to four times and generally not less than two times that of the first samples. The interval between samples two and three was 10-18 min and occurred between 34-60 min of perfusion following C^{14} glucose addition. This increase was noted with or without Nembutal or Metrazol, and the magnitude did not appear to be altered by the drugs. The striking finding of this work was the constancy of the amount and composition of the AA pool under varying conditions. The interpretation of this work and its relation to previous findings has been discussed earlier.

The concept of endogenous substrate metabolism is supported by Sutherland who found that Q_{O_2} values observed in slices of human brain cortex _in vitro_ could not be accounted for on the basis of CHO metabolism alone (57). At no time, in the presence or absence of glucose, did $C^{14}O_2$ production approximate the O_2 consumed. The endogenous Q_{O_2} of human brain could not be accounted for by CHO metabolism alone. Even if lactic acid was included in the intracellular fraction, this would account for less than one hours O_2 uptake. With 0.01 M glucose, the initial glucose uptake was in excess of requirements. Part appeared as lactic acid, but the remainder must be assumed to be stored or undergo anabolic reactions. A portion of the 'stored' amount appeared to be subsequently oxidized, since

during the first two hours of respiration glucose oxidation and lactic acid formation increased without a concomitant increase in uptake. By the end of three hours, glucose uptake exceeded that necessary to account for the total O_2 consumption suggesting that anabolic reactions involving glucose were increasing or glucose was substituting for endogenous substrates which had been depleted.

Contradictory evidence to the above ideas of neural metabolism is presented by Horowicz and Larrabee (32). Experiments dealing with the oxidation of glucose in mammalian sympathetic ganglia at rest and during activity confirmed the concept that enough glucose was oxidized to CO_2 by resting neurons to account for most all of the resting O_2 uptake. They could not confirm the necessity for the oxidation of some other substrate in addition to glucose in the presence of neuronal activity. In their preparations repetitive stimulation of preganglionic nerves consistently produced increased rates of labeled CO_2 production.

SUMMARY OF INTRODUCTION

1) The field of metabolism in the CNS is relatively new, being dependent upon recent advances in methodology for development.

2) The importance of the relationship of chemistry to function is discussed.

3) Evidence supporting the uniqueness of the dependence of CNS metabolism on an uninterrupted supply of glucose is presented. On the other hand, this does not imply that the pathways of glucose metabolism

in the brain lead directly only to oxidation. Various chemical and energy transformations between the initial energy sources, oxygen and glucose, and the final products, carbon dioxide and water, may occur so that various intermediate compounds derived from glucose or produced by energy made available from glucose catabolism may be the actual substances finally oxidized.

4) The concept of endogenous substrate metabolism and its contribution to metabolism during activity is presented and supported by the work and interpretations of Geiger and co-workers. This idea, however, is not substantiated by findings of Horowicz and Larrabee of a consistent increase in $C^{14}O_2$ levels following induced activity.

5) Evidence indicating the possible importance of tissue integrity to transfer mechanisms and metabolic processes is presented.

In view of the above concepts concerning possible differences in CNS metabolic pathways during activity and the importance of tissue integrity to substrate availability, the following experiments were conducted.

EXPERIMENTAL PURPOSE AND DESIGN

EXPERIMENTAL PURPOSE AND DESIGN

The experiments were designed to determine resting glucose uptake levels of a relatively intact preparation of central nervous system tissue, and to see if a correlation existed between induced levels of activity and glucose uptake in this preparation. It was anticipated that the preparation used in the series of experiments to be described would yield information about CNS metabolism from tissue samples in which not only cell integrity, but organ structure with intact membranes would be maintained to a degree not permissible with the usual incubation type of procedure.

The original experimental procedure consisted of perfusing an isolated frog's spinal cord with an oxygenated nutrient frog Ringers solution which was recirculated from a reservoir contained in a constant temperature water bath, through the experimental chamber, and back to the resevoir. This apparatus allowed the cord to be electrically stimulated by means of its dorsal roots, and the magnitude of its induced electrical activity recorded from the ventral roots. It was hoped that by utilizing various stimulating frequencies, the activity level of the preparation could be controlled. Samples of the perfusate would be periodically withdrawn and the concentration of glucose in these determined. Thus one would be able to simultaneously control and measure the activity level of the tissue and compare this with its glucose uptake.

With a total recirculating volume of 25.0 ml of nutrient solution, experiments could be designed in which the same preparation would be used for comparison of the effects of various frequencies of stimulation on the rate of glucose uptake. This would be possible because the large

volume allows for numerous samples to be taken during the experiment. However, as will be explained in detail later, the absolute amount of glucose contained in such a volume masked the small uptakes of glucose produced by the spinal cord preparation. It was therefore necessary to re-structure the experiment around the use of a small volume permitting only single samples.

MATERIAL AND METHODS

MATERIAL AND METHODS

Experimental chamber.

The experimental chamber in which the isolated spinal cord was contained was designed to meet several requirements. The first demand was that of providing a means for housing an isolated spinal cord in mechanical and thermal stability for long periods of time while being continuously perfused. Secondly, it must allow means for stimulating and recording. It must also provide a pathway through which an oxygenated nutrient fluid could be recirculated and a means by which samples of this fluid could be withdrawn.

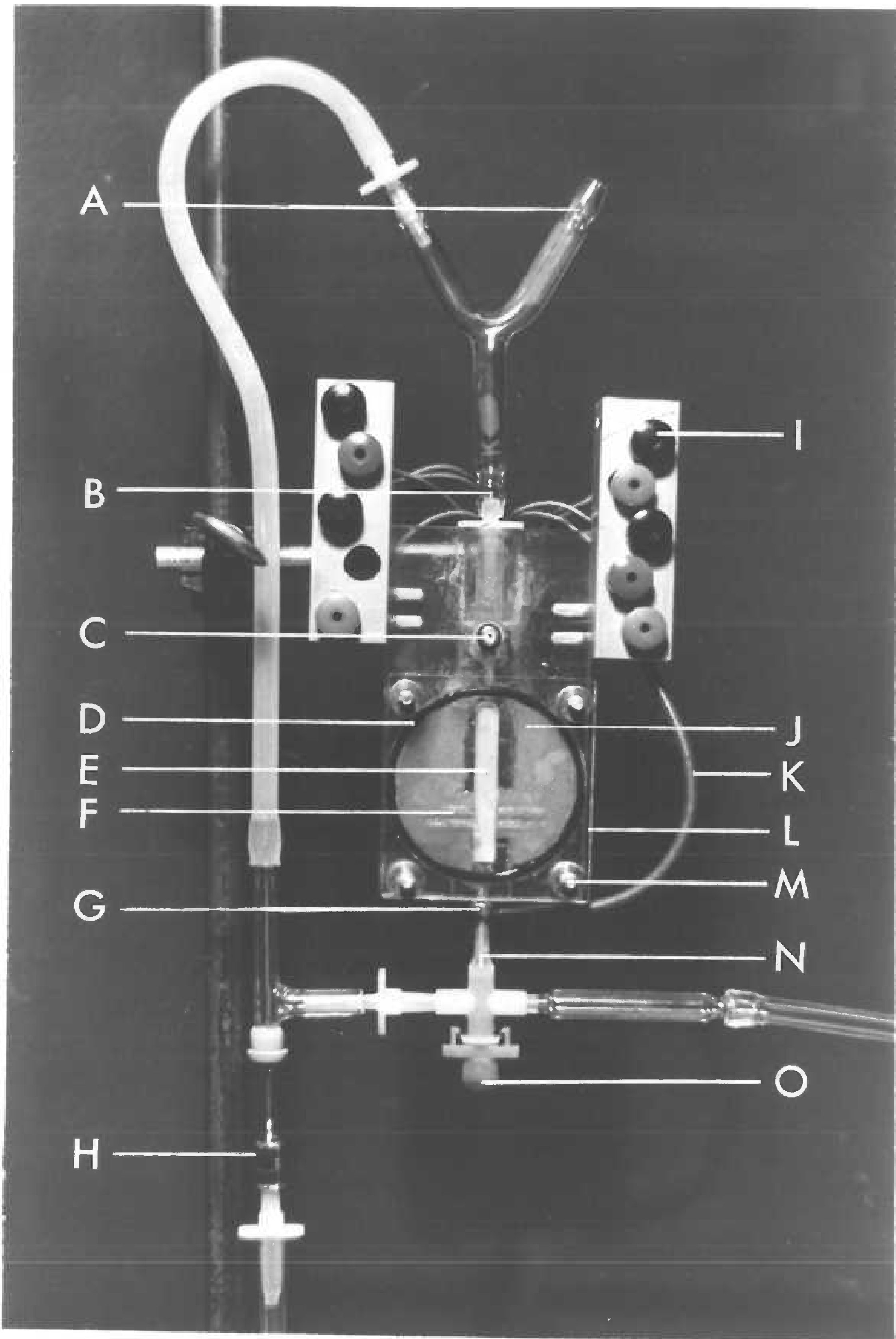
The experimental chamber (Fig. 1) consisted of a lucite block which contained a central chamber with a silicone rubber floor. This floor allowed the cord preparation to be mounted and secured by two fine pins, thus providing the mechanical stabilization needed during the experiments. Two side channels (electrode channels) branched off each side of the central chamber. In the floor of each of these, two electrodes were placed. The electrodes were made from 0.020 in. diameter platinum wire and the tips of these were shaped in the form of a "T" to provide the maximal surface area for contact. One side was used for dorsal root stimulation and the other for ventral root recording.

In the original apparatus, the nutrient fluid was recirculated by means of a Barron peristaltic pump. The portion of the recirculation

FIGURE 1

The experimental chamber with the external portion of the recirculation pathway, oxygen aeration needle, and the connector from the volumetric burette reservoir attached. The parafilm plates and chamber cover are in place. The chamber is held in the vertical position used during the timed perfusion portion of the experiments by means of a ring stand. The figure is approximately two-thirds the actual size.

A) O_2-CO_2 aeration gas outlet.
B) Chamber inlet.
C) Thermistor connection - There is a second connection for the second thermistor just opposite the connection shown, but on the reverse side of the chamber.
D) Rubber O ring placed in its groove.
E) Central tissue chamber with rubber floor.
F) Electrode side channels for dorsal and ventral roots.
G) Chamber outlet.
H) O_2-CO_2 aeration needle.
I) Stimulating and recording electrode pin jack connection.
J) Parafilm cover plates (these were made by heating two thicknesses of parafilm until they adhered, and then cutting the plates to the desired shape).
K) Preparation ground.
L) Lucite chamber cover.
M) Threaded corner posts for securing cover.
N) Three-way valve for perfusate introduction with the connector from the volumetric burette reservoir attached.
O) Rubber diaphragm for sample removal.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pathway external to the experimental chamber consisted of a volumetric reservoir flask immersed in a constant temperature water bath. This allowed a continual check on the volume of the perfusate during the experiment. From observations of such, it was found that the volume decrease exceeded the amount withdrawn in samples. This volume loss was corrected by humidifying the oxygenating gas before it passed through the perfusate. The perfusate was oxygenated in this flask, pumped from it through the experimental chamber, and then back to the reservoir thus completing the recirculation cycle. The total volume of the recirculating fluid was 25.0 ml.

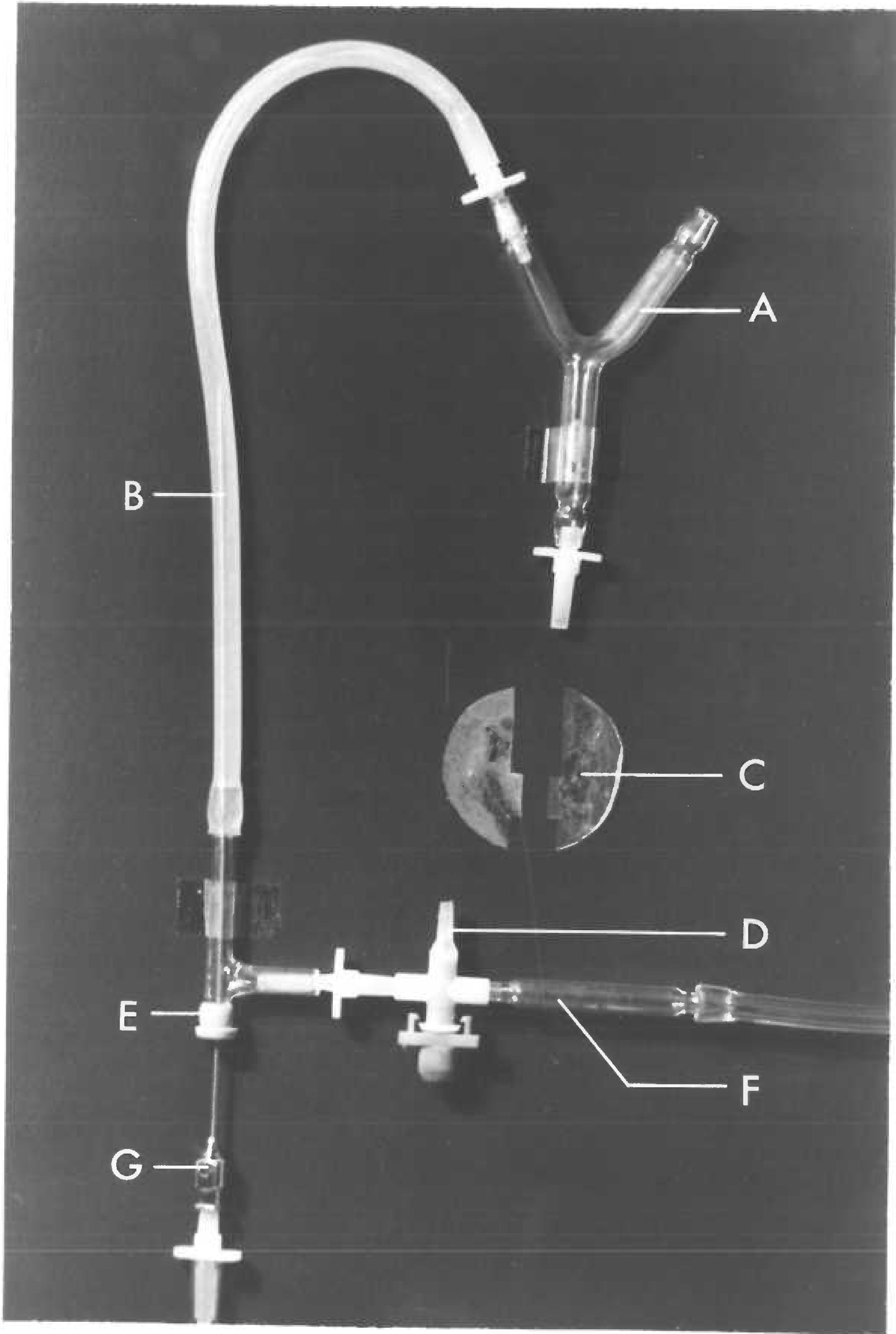
When it became obvious that the total volume of this system was too large, the external portion of the recirculation pathway and the method of maintaining perfusion was altered to reduce the total volume of recirculating perfusate (Fig. 2). This was accomplished by the elimination of the reservoir and the Barron perfusion pump. In the revised apparatus, perfusion flow was maintained by an aeration pump. The flow of the oxygenating gas mixture to the perfusion medium was utilized to carry the solution up a tygon tube situated on the lateral side of the experimental chamber and connected to the inflow and outflow of the chamber (Figs. 1 and 2). The solution then passed through the vertically oriented chamber under the influence of gravitational forces to be picked up by the gas stream and began on a new recirculation cycle.

In all experiments, the dry oxygenating gas was passed through a humidifier containing glucose-free Ringers. Thus, when the gas was introduced to the perfusing fluid, it was saturated with water vapor.

FIGURE 2

The external portion of the recirculation pathway unattached from the experimental chamber. The oxygen supply needle is inserted into its rubber diaphragm adapter and the connector from the volumetric burette reservoir is in place. Examples of the parafilm plates are shown to indicate their shape.

A) Escape outlet for O_2-CO_2 aeration gas.
B) Tygon tubing.
C) Parafilm plates.
D) Three-way valve for the introduction of perfusate from volumetric burette reservoir.
E) Rubber diaphragm for aeration needle.
F) Connector from volumetric burette.
G) O_2-CO_2 aeration needle.


A
B
C
D
E
F
G

This eliminated the possibility of the gas picking up water from the perfusate and thereby concentrating it with respect to glucose.

Samples were withdrawn through a rubber diaphragm located in the three-way valve at the outflow tract of the chamber (O in Fig. 1) by means of 1.0 cc tuberculin syringes.

In the original apparatus, an attempt was made to maintain the temperature at 18 - 20° C by placing the perfusate reservoir in a constant temperature water bath. Maintenance of constant temperature would permit quantitative comparisons between experiments. The choice of temperature was conditioned by the desire to avoid the development of an anoxic core. Larrabee has reported (38) that the anoxic core was removed even when oxygen concentrations as low as 60% were used in temperature ranges of 24 - 25° C. Amphibian tissues also operate at lower metabolic rates, and this, combined with their small size, enabled metabolic observation to be made on even the intact brain of the frog (42). Although the temperature in the water bath could be maintained at the desired levels, the temperature of the solution measured in the experimental chamber, by means of thermistors, could not be kept more than 2 - 3° below room temperature. This was due to the slow rate of perfusion and the extended period of time the perfusate was in the portion of the recirculation pathway outside the cooling bath. In view of this, no attempt was made to control temperature during the experiments after revision of the apparatus.

The lucite block also contained a circular groove for the placement

of a rubber O ring (D in Fig. 1). When the lucite cover was placed over the four threaded corner posts and secured, the O ring, with its petrolatum seal, separated the perfusion portion of chamber and completed the seal in the closed recirculation pathway.

Preliminary chamber preparation.

The groove for the O ring was filled with Dow Corning stopcock grease (silicone base lubricant) and the ring pressed into place. That portion of the block which was covered with the lid was coated with a thin layer of the same lubricant, both inside and outside the O ring groove. The openings of the electrode channels were separated from the tissue chamber by means of dams constructed with petrolatum injected at their openings from a syringe fitted with an 18 gauge needle. This prevented the entry of the Ringers electrolyte solution into the electrode channels during the cord mounting and subsequent experimental procedure. As both the stimulating and recording electrodes were permanently fixed in the floor of the channels, the only way to clean them was with a test tube brush and carbon tetrachloride. This was done three to four times before each experiment, and then the electrodes were scraped with a metal probe.

Biologic preparation.

The experiments have been carried out using grass frogs (Rana pipiens) as a source of central nervous system tissue. These were obtained during the fall and winter months. They were ordered in lots of a size to supply approximately a week's experiments and stored in a refrigerated environment (8°C) without food.

The spinal cord was removed by the method described by Brookhart, Machne and Fadiga (14). This consisted of rapid decapitation of the animals and removal of a rectangular block of tissue containing the spinal column and urostyle. The skin was removed from this block; and then with four pins it was secured, ventral side up, on the rubber floor of the dissection chamber. The chamber was continuously supplied and drained of a glucose-free frog Ringers solution by a gravity flow input and vacuum suction removal. This solution was equilibrated with a 95% O_2 - 5% CO_2 gas mixture. Then, with the aid of a dissecting microscope, starting at the urostyle, the ventral portion of the spinal column was removed with the dura thus exposing the spinal cord and roots. Spinal roots 9 and 10 were dissected free to be removed with the cord. They were used for dorsal root stimulation and ventral root recording because of their convenient length. The remainder of the roots were cut at their emergence from the cord. The cord was freed from the block and the anterior portion of the cord severed just cephalad to the brachial plexus in an attempt to standardize the preparations.

Preparation transfer and mounting.

The cord, having been removed from the tissue block, was then transferred and fixed to the silicone block in the floor of the experimental chamber by means of a pin which had been placed transversely through its upper portion. It was mounted lateral side up, and the dorsal and ventral roots of segments 9 and 10 severed just proximal to the dorsal root ganglion allowing the longest possible length of the

roots. The roots were then placed over the respective stimulating and recording electrodes by grasping their severed distal ends with fine tipped forceps and laying them over the electrode tips. Proximal to the root contact with the proximal electrode the roots were gently pressed into the petrolatum dams at the openings of the channels. Once a satisfactory and maximum response was obtained and visualized on the oscilloscope, the roots were covered with petrolatum and the side channels filled with the same substance. This isolated them in a non-conducting medium during the experiment and prevented them from drying. The caudal end of the cord was then secured with the second pin.

Parafilm plates (J in Fig. 1 and C in Fig. 2) were placed over the lateral portions of the recording chamber inside of the O ring and carefully pressed into position thus sealing this portion of the chamber and the electrode side channels and their contained nerve roots from the electrolyte Ringers solution. The medial edges of these plates were compressed against the chamber and sealed with petrolatum.

During the mounting procedure, the cord was bathed with the glucose-free dissecting Ringers. Just prior to the transfer of the cord, the gravity flow feed of Ringers solution was changed from the dissection chamber to the experimental chamber. Flow from the chamber was maintained by vacuum suction. This method of perfusion was continued until the parafilm plates were placed, sealed, and the chamber preparation was ready for the cover to be placed and secured. At this point, the perfusion inflow was shut off and detached from the experimental chamber. The oxygen supply was diverted from the dissection Ringers to the experimental

chamber by means of a three-way valve. The chamber was rotated from a horizontal to a vertical position and completely evacuated of glucose-free dissection Ringers by the vacuum suction, which was then detached. The external portion of the recirculation pathway of the chamber was connected, and the 8 in. 18 guage needle through which the oxygen was supplied to the chamber inserted into its rubber diaphragm adapter. Next, the cover was placed and secured. Three milliliters of the glucose-Ringers was introduced into the chamber from a 10.0 ml volumetric reservoir burette through a three-way valve located at the outflow of the chamber. The oxygen aeration needle was lowered into the perfusion medium; and by it's height and control of the gas flow, the perfusion rate of the preparation was regulated. This point marked the beginning of the timed perfusion period of the experiment.

Perfusing solutions.

The perfusion solution used in the experiments was that described by Brookhart (14). The solution in ionic concentration is similar to frog plasma. The ionic concentrations in millimoles per liter were: NaCl 72.6, KCl 3.08, $NaHCO_3$ 34.85, $CaCl_2$ 1.54, and a phosphate buffer to pH 7.4 when equilibrated with the gas mixture of 95% oxygen and 5% carbon dioxide. For details of the method of preparation and storage of the solutions see appendix A. From the basic frog Ringers solution two different perfusing solutions were made for the experiments:

1) a dissecting glucose-free Ringers solution,

2) a glucose-free Ringers solution for perfusion from which the various concentrations of nutrient glucose-Ringers were prepared.

The dissecting Ringers was used during the dissection and the transfer and mounting of the tissue preparation. This fluid was not recirculated.

The glucose concentrations used for the nutrient perfusates varied from 0 - 300 mg%. The majority of the experiments were carried out with concentrations of 100 mg%. This level is approximately 2.5 times that of frog blood sugar concentrations (18). A series of experiments were conducted in which the cord preparations were perfused with solutions which contained no exogenous source of a metabolic substrate.

With the original apparatus, the total recirculating volume during the experimental procedure was 25.0 ml. The change of the method of perfusing allowed this volume to be reduced to 3.0 ml.

As will be explained in detail later, the experimental chamber became contaminated with micro-organisms. To eliminate this factor, penicillin and chloromycetin were added to the perfusing fluid. Details concerning their concentrations are presented in appendix A.

Stimulation.

Dorsal roots 9 and 10 were simultaneously stimulated in all experiments to activate as large a portion of the spinal cord motor neurons pool as possible. Stimuli consisted of rectangular pulses with a duration of 0.2 msec and an amplitude of 15 - 20 volts. They were delivered from a Tektronix Type 161 Waveform Generator and Type 162 Pulse Generator and passed through an Argonaut LIT 069 Isolation Transformer. The output of the isolation transformer was measured at 2 - 3 volts.

Such stimuli were always supramaximal.

Recording.

Recordings of the ventral root discharge were amplified by means of a Tektronix Type 122 RM Low-Level Preamplifier. This preamplifier allowed selective rejection of unwanted electrical interference. The tissue preparation was grounded through a metal tube which served as the outflow tract of the chamber, and all experiments were conducted in a grounded wire cage to further eliminate 60 cycle interference.

The response was then simultaneously monitored on channel A of a Tektronix Type 502 Dual-Beam Oscilloscope, on the upper channel of a two channel Grass Polygraph write out system, and introduced to the Grass Instrument Company 5U-1 Integrator Pre-Amplifier (see block diagram in Fig. 3). Thus, both a permanent write out record and a visual monitor of each individual response was provided.

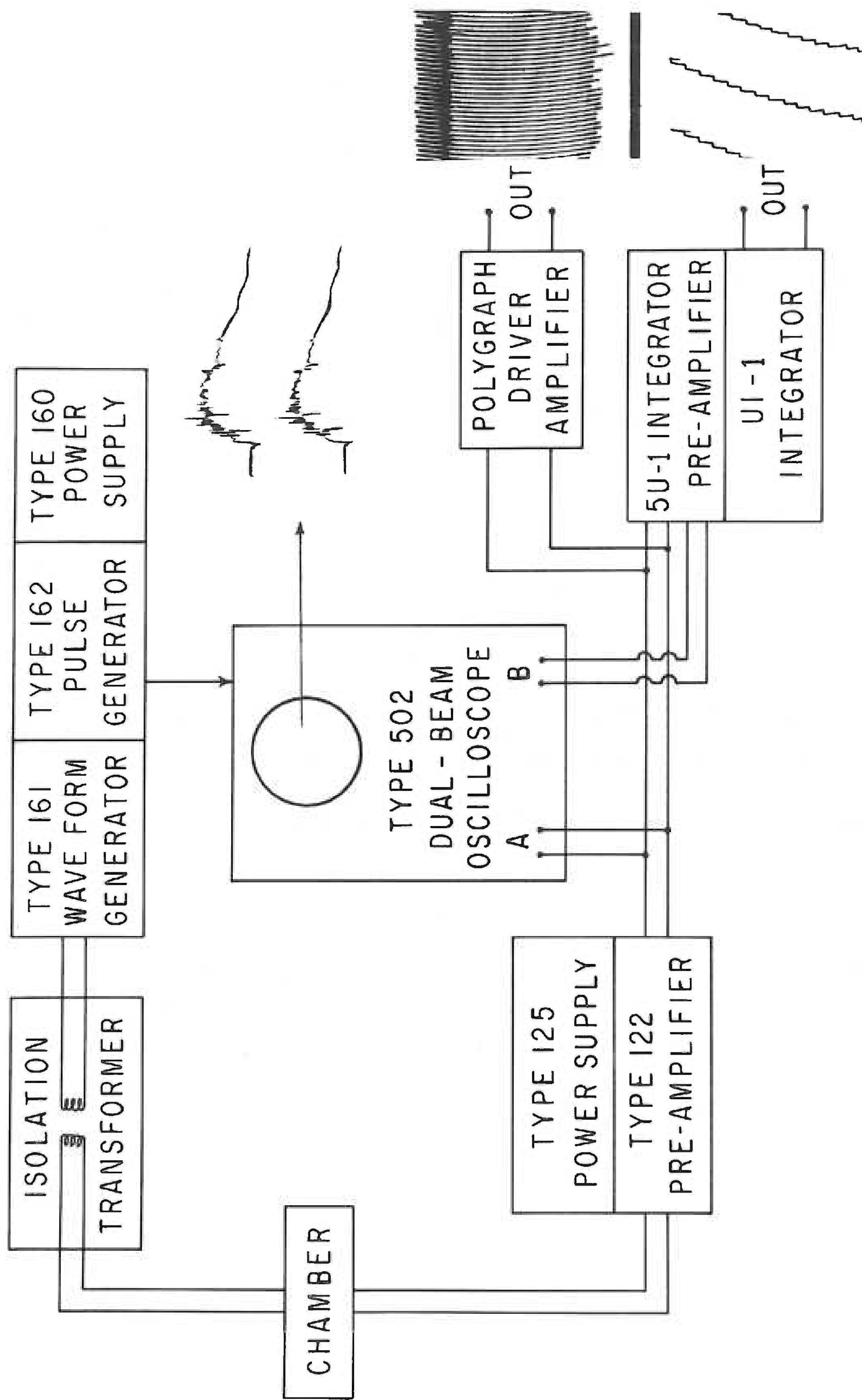
The output of the 5U-1 Pre-Amplifier, which represents the signal to be integrated, was monitored visually on channel B of the dual-beam oscilloscope. Therefore, by comparison of the two responses on the oscilloscope, it was possible to adjust the output of the integrator pre-amplifier to a level where the signal to be integrated matched the amplified response recorded from the cord preparation (see Fig. 3 for example).

Determination of the electrical output of the spinal cord preparation.

The Grass Instrument Company UI-1 Unit Integrator and 5U-1 Integrator

FIGURE 3

A block diagram of the experimental apparatus. Also shown are examples of the response as visualized on the dual-beam oscilloscope and as recorded by the polygraph write out.



TYPE 160
POWER
SUPPLY
TYPE 162
PULSE
GENERATOR
TYPE 161
WAVE FORM
GENERATOR
ISOLATION
TRANSFORMER
CHAMBER
TYPE 502
DUAL - BEAM
OSCILLOSCOPE
A
B
TYPE 125
POWER SUPPLY
TYPE 122
PRE-AMPLIFIER
POLYGRAPH
DRIVER
AMPLIFIER
OUT
5U-1 INTEGRATOR
PRE-AMPLIFIER
UI-1
INTEGRATOR
OUT

Pre-Amplifier were utilized in an attempt to measure the total accumulative electrical output of the ventral root discharge of the cord preparation. The total output evoked by each stimulus is a function of both the amplitude and the duration of the response. Therefore, measurement of this characteristic involves measurement of the area of the voltage-time curve.

The method consisted of recording the definite integral (total accumulated area) of each response on a Grass Model 5 Polygraph write out system. The definite integral of each individual response was represented by a vertical deflection of the stylus, the height of which was proportional to the total area of the response. The stylus was maintained at this level until the next response was received by the integrator at which time its area was recorded by a second vertical deflection. Thus, a ramp was established; the slope of which was a function of the area and frequency of a succession of responses. The height of the ramp was determined and set by the automatic or manual reset controls of the integrator. Every time the Unit Integrator is reset, its memory of past accumulated area is destroyed and set to zero, and a new time cycle is begun in which the time is set to zero at the beginning of the cycle. One such cycle is termed an epoch, and epoch time or epoch length refers to the time between successive cycles.

By comparing the mean epoch time of an experimental procedure with that of an epoch derived from a known voltage source, it was possible to calculate the quantity of "electricity" required to produce the

experimental epoch. Then, by multiplying this value by the number of corrected total epochs[1] produced during the timed experimental period of stimulation, the total accumulated electrical activity produced by the cord was calculated.

Calibration of Integrator.

The Grass Driver Amplifier, 5U-1 Pre-Amplifier and Unit Integrator were calibrated as specified by the manufacturer. With the amplifier gain, integrator sensitivity and clipping level of the 5U-1 set at levels suitable for recording the response discharge from the biological preparation of each individual experiment, a one volt peak-to-peak signal was taken from the calibration output of the 502 Dual Beam CRO, passed through a variable resistor, and fed to the input of the 5U-1 Pre-Amplifier. This signal had a frequency of 1000 cps and a duration of 0.5 msec. It therefore is equivalent to an arbitrary value of 500 mV-sec. when allowed to run for one second (1000 mV x 1 sec x 0.5 duty cycle). The variable resistor in this circuit allowed the alteration of the one volt signal to various levels before introduction to the 5U-1. By adjusting the amplitude of this signal, which was monitored on the oscilloscope for accurate calibration and simultaneously on the polygraph write out system after integration by the UI-1 Integrator, a calibration source, with a

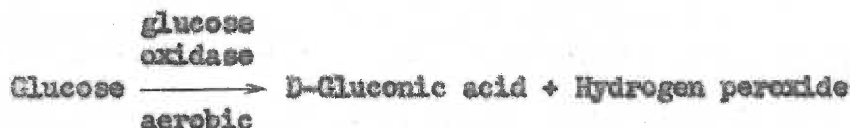
[1]Corrected total epochs refers to the number of epochs actually resulting from cord activity and is derived by subtracting the amount of "noise epochs" from the actual number of epochs recorded in the timed interval. The origin of this noise remained undetermined, but was presumed to be from poor contact of nerve roots on the recording electrodes. The magnitude of the noise element was determined by recording the output from the system during timed periods with the stimulus off. From these records, a mean slope and noise epoch length was obtained and the number of noise epochs introduced into the record calculated.

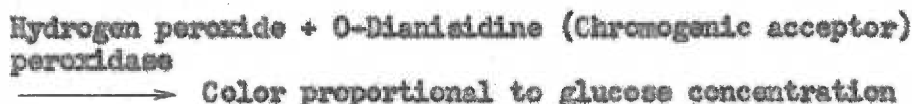
range of 0 - 500 mV-sec, was made available from which a quantitative evaluation of the electrical response of the tissue preparation could be made. At least three calibration voltage levels were used for each experiment. From their epoch times a graph[2] was made and used to quantitate the mV-sec contained in the mean corrected experimental epoch.

Glucose determinations and sampling technique.

The method of glucose determination chosen for this series of experiments consisted of the enzymatic oxidation of glucose to gluconic acid. This procedure provided specificity and sensitivity not available with determinations based on reducing methods and in addition was time saving.

The method is based on the almost specific enzymatic oxidation of glucose by glucose oxidase, which has been found to be a typical aerobic dehydrogenase. The end products of this oxidation are gluconic acid and hydrogen peroxide. Since both of these end products are colorless, the hydrogen peroxide then reacts with a chromogenic hydrogen donor in the presence of peroxidase and the yellow color formed at a given time is proportional to the concentration of glucose originally present (34, 51).





Glucose oxidase has been shown to be relatively specific for beta-glucose

[2] It was found that by plotting the epoch time (in sec) against the calibration voltage level (in mV) on logarithmic paper, straight lines were obtained.

oxidation (36). The sensitivity and variability of the method was determined and checked by several means. The methods used, results of the procedures, technique of the determinations, and other characteristics of the method are given in detail in appendix B.

It was decided from the results of the sensitivity determinations that the method could detect differences in concentration of 2.5 μg/ml. Thus, in all experimental determinations, the solutions tested were considered to be of the same concentration unless this difference existed.

The reduction in the volume of the perfusing fluid from a 25.0 ml reservoir to 3.00 ml total was an attempt to reduce the absolute amount of glucose available to the tissue for metabolism to a level where small uptakes by the tissue would be reflected in concentration changes of a magnitude detectable by the enzymatic method. For example, using as a reference the uptake rates found by Rafaelsen (46) with isolated pieces of rat spinal cord (2.0 ± 0.14 mg glucose/gram of wet spinal cord/hour with a 75 mg% glucose concentration in the medium), the total glucose uptake for a 120 minute period would be 200 μg for a spinal cord of 50 mg weight. This uptake reduces the amount of glucose in 25.0 ml of 100 mg% glucose from 25,000 to 24,800 μg which is equivalent to a concentration change from 100 to 99.2 mg% or a difference of 8 μg/ml in the undiluted samples. However, as will be explained, samples had to be diluted to a concentration of approximately 10.00 mg% to allow a readable range on the spectrophotometer. This dilution factor of 11.0 reduces the concentration change to one of from 9.09 to 9.02 mg% or

0.7 μg/ml in the samples on which the determinations were run.

$$\frac{100 \text{ mg\%}}{11} = 9.09 \text{ mg\%} \qquad \frac{99.2}{11} = 9.02 \text{ mg\%}$$

This value lies well within the range of variability of the method of determination used.

An uptake of 200 μg from a volume of 3.0 ml of 100 mg% solution reduces the total amount of glucose from 3000 to 2800 μg, or a concentration change of 100 - 93.3 mg%. When 1.0 ml aliquots of this are diluted with 10.00 ml distilled water, this leaves a difference in concentration of 9.09 to 8.48 mg% or 6.1 μg/ml which is detectable by the glucostat method.

$$\frac{100 \text{ mg\%}}{11} = 9.09 \qquad \frac{93.3 \text{ mg\%}}{11} = 8.48$$

Another alternative would have been to use undiluted samples for the glucose determinations. Attempts at this met with failure for unknown reasons. If the optical density reading of a sample falls on the right half of the OD scale, one is supposed to be able to advance the sensitivity of the spectrophotometer, and for each scale advancement add 0.5 OD units to the reading obtained to convert the reading to the original sensitivity base at which the machine was standardized with the blank sample of the determination (12). This allows the OD readings on samples of concentrations much greater than the standard to be made in the more precise portion of the scale. Several attempts at this

procedure met with failure for unknown reasons. Samples of solutions containing glucose in the range of 90 - 100 mg/100 ml were tested undiluted and diluted by the factor of 11 as were experimental samples. The OD readingsof the undiluted samples when converted by the above method gave calculated concentrations in the range of 30 - 50% of the mixed concentrations.

Also included in appendix B is a description of the methods by which samples were taken from both the large and small volume recirculation systems and the details of their treatment before the glucose determinations were run.

Glucose-1-C^{14} experiments.

During the course of the experiments, it was found that microbial contaminants were probably influencing the results of measurements of glucose uptakes. Steps were taken to eliminate this source of error. In the absence of such contaminants, it was found to be impossible to detect any glucose disappearance from the 25.0 ml circulating volume. Accordingly, in an attempt to determine if the preparation was metabolizing glucose, a sample of glucose-1-C^{14} was introduced into the reservoir of the recirculation pathway. The gas outlet of the otherwise air tight recirculation system was connected by polyethylene tubing to a reception flask containing NaOH. Thus, all gas introduced into the system from the oxygenating source, and any gaseous products resulting from spinal cord metabolism would pass through the NaOH which would absorb the contained CO_2. Following completion of the timed experimental period of stimulation, the collection flask was replaced and the remaining

perfusate acidified with H_2SO_4 to release all dissolved CO_2. This would then be absorbed in the second collection flask. Radio-activity found in the two collection flasks as labeled CO_2 would indicate that the cord had metabolized a portion of the introduced glucose-1-C^{14}. By use of the ratio of labeled glucose to the total amount of glucose present in the perfusate, the amount of glucose aerobically metabolized to CO_2 by the cord could be calculated.

Correlation of electrical and glucose measurements.

The total electrical output of the cord preparation, expressed in volt seconds (V-sec) was correlated to the total glucose disappearance from the perfusate during the timed period of stimulation. This was done by two methods:

1) Graphically by plotting the total glucose disappearance against the total electrical output; and,

2) By calculating the micrograms of glucose required to produce one V-sec of electrical activity.

Such correlations are not without limitations. It was easily demonstrated that the nature, amplitude, and therefore area of the discharge response could be altered by several factors. These are listed below in their apparent order of significance.

1) Adequacy of contact between the ventral root and recording electrode.

2) Position of the recording electrode on the ventral root with respect to the distance from the cord.

3) Viability level of the cord preparation.

Attempts at controlling these modifying factors met varying degrees of success.

The best contact of the nerve root was obtained by repositioning the root over the electrodes until the largest response was obtained. Then the root and side channels were covered and sealed with petrolatum.

The amount of the electrotonically propagated ventral root slow wave recorded in the ventral root discharge is dependent upon the distance of recording electrode from the cord. Proximity to the cord allows this component to be recorded as it forms a major portion of the discharge, whereas distal displacement minimizes the contribution of the slow wave to the recorded ventral root response (14). For each experiment, all possible combinations of recording points were examined; the response with the largest area and the least amount of background noise was chosen to be recorded during the experiment.

The viability of preparation was preserved by rapid, but careful, removal from the animal and transfer to the experimental chamber. No preparations were used unless the initial response had an amplitude of at least 0.5 mV as witnessed on the CRO.

Therefore, for each preparation, the response chosen to represent the total electrical output of the cord was the largest one available with the least amount of background noise. What proportion of the true total output this represented was never known.

Attempts to correlate the glucose uptake of the preparation on a tissue weight basis, using the frequency of stimulation as the sole

grouping determinant, is also severely limited in regards to validity. It is apparent from the data that with the wide range of response amplitudes obtained, the frequency of stimulation was not directly proportional to the total recorded electrical output. A preparation with a recorded response amplitude of 4.0 mV at a stimulus interval of 4000 msec would have an electrical output comparable to a preparation with a 1.0 mV response at a stimulus interval of 1000 msec. A second factor limiting this comparison was the inability to determine what percentage of the total cord weight was being activated by stimulating only dorsal roots 9 and 10 on one side of the cord.

Nevertheless, in spite of these inherent difficulties, the results obtained did indicate possible trends. However, their interpretation must be made with the noted limitations.

RESULTS

RESULTS

Control of Micro-organism contaminants and the effects of their metabolism.

A series of experiments was designed to run for extended periods of time to allow the accumulative glucose uptake of the spinal cord to reach levels of magnitude detectable by the glucose oxidase method. These preparations, while being continuously perfused with 25.0 ml of glucose-Ringers, were stimulated at constant frequency until a response was no longer obtainable. The duration of these experiments, determined by the failure of the preparation to respond to the continuous stimulation, ranged from 15-18 hours.

Results from three experiments (Exp. 36, 37, and 38) with the stimulus interval set at 10,000 msec indicated relatively similar slopes when the total disappearance of glucose from the perfusate was plotted against the time of the experiment (Fig. 4).

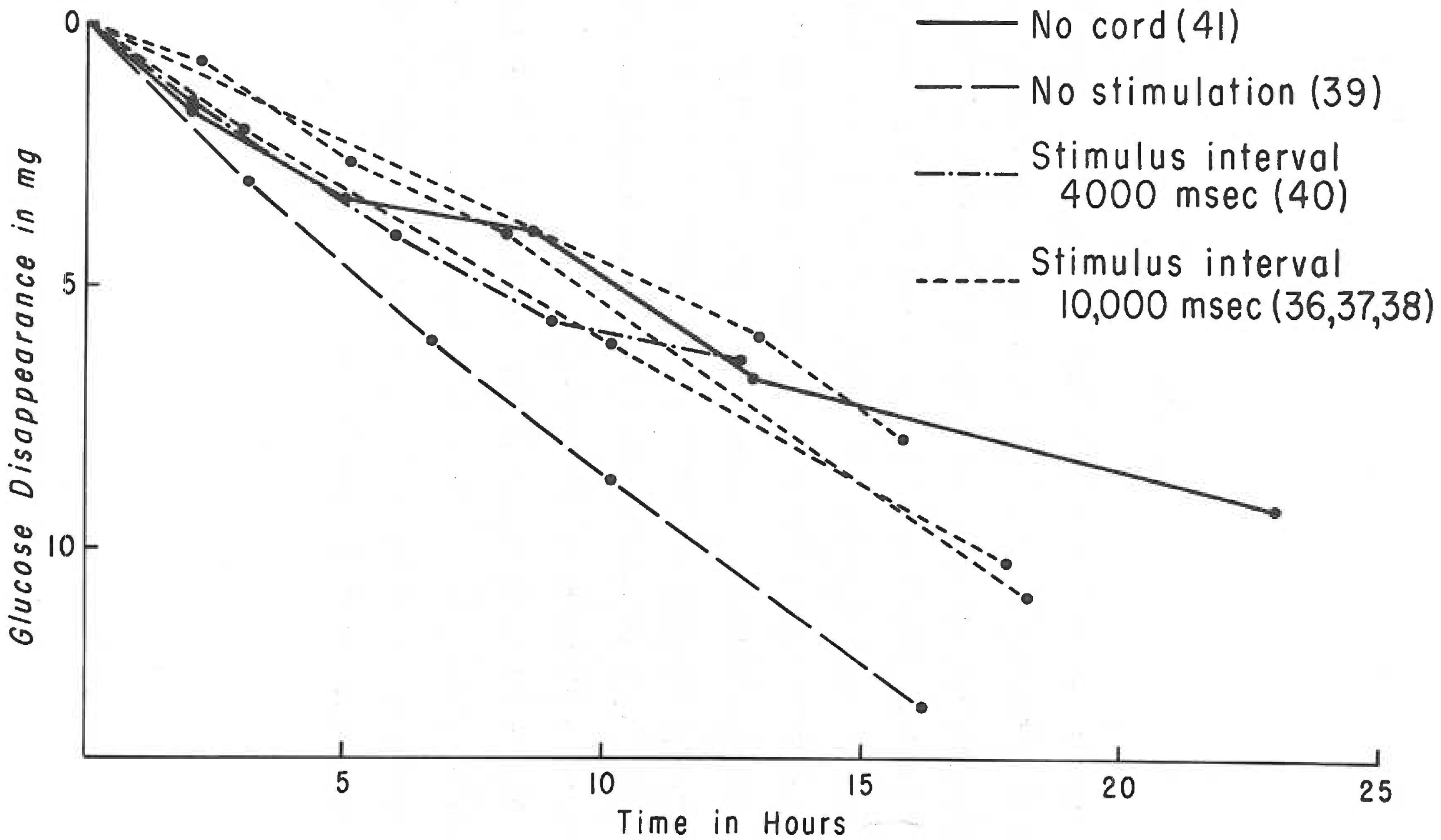
In an attempt to determine the effect of various frequencies of stimulation on the glucose uptake, an experiment (Exp. 39) in which the preparation was not stimulated, and an experiment (Exp. 40) in which the stimulus interval was reduced to 4000 msec were carried out. The resultant uptake slopes closely resembled those of the three previous experiments at a stimulus interval of 10,000 msec (Fig. 4). This indicated that the glucose uptake of spinal cord tissue was independent of induced activity levels, or that the glucose disappearance from the perfusate was dependent upon some other factor than the spinal cord preparation.

FIGURE 4

Results of a series of experiments designed to allow possible glucose uptakes by the spinal cord preparation to accumulate to levels detectable by the method of glucose determination used. The total glucose disappearance from the perfusate is plotted against the time of the experiment. The points on each line indicate the times at which samples were taken for glucose determinations.

It was noted that the results represented in this manner indicated that frequency of stimulation, representing the level of activity of the preparation, had little effect on the glucose disappearance detected during the experiment.

The results of experiment 41, in which no cord preparation was placed in the chamber, confirmed the possibility that glucose disappearance was independent not only of the induced level of activity, but also of the biologic preparations.


No cord (41)
No stimulation (39)
Stimulus interval 4000 msec (40)
Stimulus interval 10,000 msec (36,37,38)
Glucose Disappearance in mg
0
5
10
Time in Hours
5
10
15
20
25

Accordingly, an experiment (Exp. 41) was run in which no spinal cord was placed in the chamber and 25.0 ml of 100 mg% glucose-Ringers was recirculated for a period of 23 hours with samples taken for glucose determinations at various time intervals. The results of these determinations are compared with those obtained in experiments 36-40 in Fig. 4. From this, it was concluded that the frog spinal cord contributed relatively little to the disappearance of glucose from the perfusate.

Since the uptake or disappearance slope seemed to remain constant throughout the duration of the experiment, bacterial and/or mycotic contamination of the apparatus was considered as the most probable source of the glucose uptake.

Experiment 43 was essentially a repeat of the "no cord" experiment except for the following changes which were made in an attempt to eliminate possible micro-organism contaminants.

1) All polyethylene and tygon tubing was replaced.
2) All tubing adapters were placed in 70% isopropyl alcohol for 12 hours.
3) 0.25 ml of 100 mg% chloromycetin was added to the perfusate.

The experiment consisted of recirculating 25 ml of glucose-Ringers, with the added antibiotic, through the empty chamber for 19 hours with samples taken at the timed intervals. The results of these determinations are shown in Table 1 and indicate essentially no glucose disappearance.

TABLE 1

Glucose determinations from experiments in which no cord preparation was placed in the experimental chamber. The perfusate consisted of 25.0 ml of 110.0 mg% glucose-Ringers with 0.25 ml of 100 mg% chloromycetin added. Standard was 110.0 mg% glucose, without chloromycetin, diluted by the factor of 11 (10.00 mg%).

Time interval between samples:

Sample I	:	beginning of experiment
Sample I-II	:	3 hours
Sample II-III	:	4 hours, 15 minutes
Sample III-IV	:	4 hours, 30 minutes
Sample IV-V	:	7 hours, 10 minutes

The calculated concentrations indicate that the attempt to sterilize the perfusate and the experimental apparatus greatly reduced, if not eliminated, the glucose disappearance noted in the previous experiments.

TABLE 1: Exp. 43; Sept 26.

Sample		OD Reading		OD Ave.	Calculated Concentration mg%
Std Std		193 194	191 192	192	10.00
A	I	200	196	196	10.21
B C	II*	200 197	196 194	195	10.16
D E	III*	198 193	195 192	194	10.10
F	IV	192	194	194	10.10
G H	V*	190 189	189 189	189	9.84

*Paired samples were taken at each of these times and the OD Readings indicated as the average of the two.

Therefore, the possibility that microbial metabolism had accounted for the major source of the glucose disappearance was highly suggested.

Further evidence bearing on the problem of bacterial metabolism was sought as it was decided that bacterial metabolism could have accounted for the glucose uptake rates found in the previous experiments. An experiment was set up to allow culture samples to be taken from the perfusate (not containing any antibiotic), after it had made one passage through the chamber, and additional samples after 15 minutes, 5 hours, and 10 hours of perfusion time. Samples of 0.1 and 0.2 ml were plated on both Sabouraud's and infusion agar plates and these allowed to incubate 48 hours.

The culture of the perfusate, after its initial passage through the chamber, showed heavy growth on both the infusion and Sabouraud's agar. These were identified by colony characteristics as being both bacteria and algae. Their number was such that it was impossible to count or to attempt to identify them. The contamination did not seem to increase significantly in amount with time. It therefore seemed that the contaminating organisms were present in the system and were not increasing in number during the experiment.

An attempt was made to eliminate this problem of contamination. The procedure was repeated using a glucose-Ringers perfusate which was first passed through a microfilter, and to which 20 units of penicillin per ml was added (500 units per 25.0 ml). Samples of 0.1 ml were taken

at time intervals of 0, 5 min, and 9 hours and again plated on infusion and Sabouraud's agar. Only one colony appeared on the 0 time plates (this perfusate had not passed through the chamber). However, contamination on the 5 min and 9 hour plates remained moderate. There was no sign of algae. Gram stains of five different colonies revealed the organisms to be various gram negative rods and one a possible gram negative cocci.

An infusion broth culture was made with samples of all types of colonies present being included. This was then plated on infusion agar for antibiotic sensitivity tests. Antibiotics used were:

Penicillin	Vancomycin
Streptomycin	Tetracycline
Erthromycin	Bacitracin
Novobiacin	Ristocetin
Neomycin	Staphcillin
Chloromycetin	

The organisms were shown to be sensitive to chloromycetin (5 microgram disc) and partially to tetracycline (5 microgram disc). On the basis of these findings, it was decided in all further experiments to:

1) Include 5 µg of chloromycetin in the perfusate.

2) Reduce the penicillin to 10 units/ml.

3) Filter the perfusate prior to each experiment.

4) Pump 70% isopropyl alcohol solution through the tubing and chamber for 20 minutes following the completion of each experiment.

5) Place all adapters and fittings in a similar solution between experiments.

6) Periodically run experiments in which the chamber would be perfused with glucose-Ringers in the absence of a spinal cord; to check for bacterial contamination by glucose determinations on starting and ending samples.

It was later discovered that it was not necessary to filter the perfusate since this was not a source of contamination. In addition, after several weeks of cleaning the apparatus with the 20 minute alcohol flush, the gradual and progressive appearance of cracks in the lucite chamber was detected. In view of these observations, both procedures were discontinued.

Glucose-1-C^{14} Experiments.

Two experiments were conducted with radioactively labeled glucose to determine if the cord preparation was metabolizing the glucose present in the perfusate.

1. Experiment 56: Nov 8.

The preparation was stimulated for 90 minutes at a stimulus interval of 10,000 msec. The perfusate consisted of 100 mg% glucose solution with the added antibiotics, and contained 4.3 µc glucose-1-C^{14} (equivalent to 0.055 mg glucose). During the experimental period, 0.033 µc of $C^{14}O_2$ were produced. This was representative of 0.76% of the injected dose (0.033/4.3). The acidified perfusate at the termination of the experiment contained 4.13 µc of the original dose. Therefore, from these two sources, 96.8% of the injected dose was recovered. The cord preparation contained

radioactivity (134 cpm); but since a counting efficiency for the whole cord preparation was not known, this data could not be interpreted quantitatively.

By assuming a utilization rate of 0.76% for the total amount of glucose available to the tissue in the perfusate (25.0 mg), the cord preparation would have metabolized 190 µg glucose in the 90 minute period. This is equivalent to an uptake rate of 1.71 mg glucose/gm wet wt/hr. This rate represents only the amount of glucose metabolized with the ultimate production of CO_2 and does not include that amount incorporated into tissue components or only metabolized to lactate. It has been reported that about 60% of the carbon of uniformly labeled glucose is released as CO_2 (41).

2. Experiment 58: Nov 14.

This preparation was stimulated for 120 minutes at a stimulus interval of 500 msec. The perfusate was a 300 mg% glucose solution, with the added antibiotics, and contained 5.0 µc glucose-1-C^{14}. 0.029 µc $C^{14}O_2$ were recovered during the period of stimulation and the acidified substrate yielded 3.35 µc. This represented a recovery of 67.0% of the injected dose. The spinal cord contained 88.8 cpm.

In this experiment, 0.58% of the test dose was recovered as labeled $C^{14}O_2$ produced during the period of stimulation. As 75.0 mg of glucose was contained in 25.0 ml of the 300 mg% perfusate, this indicated that the cord preparation would have metabolized aerobically 435 µg glucose during the 120 minute period of stimulation. This

is representive of an uptake rate of 3.09 mg glucose/gm wet wt/hr.

The failure of the recovery of 1.62 µc or 32% of the injected dose remained unexplained. Possible sources of the loss would include the loss of CO_2 from the apparatus, or errors in the procedures of collection, preparation, and counting of the samples. As mentioned above, the cord preparation contained detectable radioactivity but the amount was not felt to be of a magnitude adequate to account for the loss noted.

3. Interpretation:

No quantitative statements about glucose uptake rates can be made from these two experiments. However, they do indicate two important findings:

1) The spinal cord preparation was metabolizing exogenously supplied glucose with the production of CO_2. The rate of this metabolism was not far from rates observed in similar mammalian preparations (48), or from rates found later in the experiments to be described.

2) A portion of the glucose was incorporated into tissue components by metabolic processes, or was physcially absorbed by the tissue preparation during the perfusion period.

Comparison of glucose uptake and induced activity levels.

The following data represent the results of experiments conducted following the change of the external portion of the recirculation

pathway of the apparatus. The perfusing medium in all experiments consisted of a 3.0 ml total volume of a 100.0 mg% glucose-Ringers solution containing the added antibiotics. Every third to fourth experiment was run without a cord preparation to check for microbial contamination. Glucose uptakes were not observed in any of these controls. The duration of the period of stimulation was maintained at 120 minutes at the various frequencies indicated.

In table 2, the glucose uptake in mg/gm wet wt. of tissue/hour is compared for various activity levels induced by stimulating at different frequencies. The mean uptake rate for resting cord preparations was found to be 1.53 mg/gm wet wt/hour. This was not significantly different from the mean uptake rate (1.93 mg/gm wet wt/hour) found with the preparations stimulated at a 1000 msec interval ($P>0.50$)[1]. However, when the mean uptake rates of the preparations stimulated at a 4000 msec interval were compared to those stimulated every 1000 msec (0.63 and 1.93 mg/gm wet wt/hour respectively), a difference was obtained which was significant at the 15% level of confidence ($0.10<P<0.25$). The limitations of the comparison have been previously described. In these comparisons, the sole criterion for the level of tissue activity was the frequency of stimulation. No consideration of the measured electrical output was included.

[1]The Student T test was used for the statistical analysis.

TABLE 2

Results of experiments in which the glucose uptake rates, determined on a mg/gm wet wt/hour basis, are compared to the frequency of stimulation.

TABLE 2

No Stimulation		Stimulus interval 4000 msec		Stimulus interval 1000 msec	
Exp. No.	Glucose uptake mg/g wet wt/hr	Exp. No.	Glucose uptake mg/g wet wt/hr	Exp. No.	Glucose uptake mg/g wet wt/hr
78	2.00	63	0.62	67	2.37
79	1.80	64	0.00	68	2.08
81	1.63	76	1.22	69	0.00
83	0.79	77	0.00	72	1.00
87	1.41	84	1.29	73	4.22
mean uptake rate 1.53 ± 0.42		0.63 ± 0.56		1.93 ± 1.42	

Table 3 and the graphs in Fig. 5 represent data from the same group of experiments. In Fig. 5 the total electrical output during the period of stimulation for each experiment is compared to the total glucose uptake for the same period (graph A) and to the glucose uptake rate in mg glucose/gm wet wt/hr (graph B). Calculation of the correlation coefficient of the relation between the total glucose uptake and the total electrical output for the combined stimulated groups (those stimulated at a 1000 msec interval plus those stimulated every 4000 msec) yielded an r of 0.87. This is equivalent to a coefficient of alienation of 0.5 which is interpreted as leaving 50% of the variation of the data unaccounted for by any probable correlation. Taking the two groups separately, the correlation coefficient was 0.91 for the group stimulated every 1000 msec and 0.60 for the group stimulated 4000 msec. Therefore, no significant correlation between the total glucose uptake and the total electrical output, as determined in these experiments, could be found. Yet, in the group stimulated at 1/sec there is evidence of a relationship. Calculation of correlation coefficients for the comparison of the total electrical output to the glucose uptake rates yielded r values in the same range as those found with the comparison to the total glucose uptake.

This lack of correlation was also indicated when the amount of glucose uptake equivalent to the production of one V-sec of activity was determined. These values range from 0 - 1050 μg (Table 3). The only relatively consistent finding observed in the results of these experiments was that with increased frequencies of stimulation both the total glucose uptake and

TABLE 3

Results of the same group of experiments listed in Table 2 with the total glucose uptake for the period of stimulation compared to the total electrical output. Also listed for each experiment is the amount of glucose equivalent to the production of one V-sec of electrical activity, the glucose uptake rate, and the beginning and ending response amplitudes.

TABLE 3

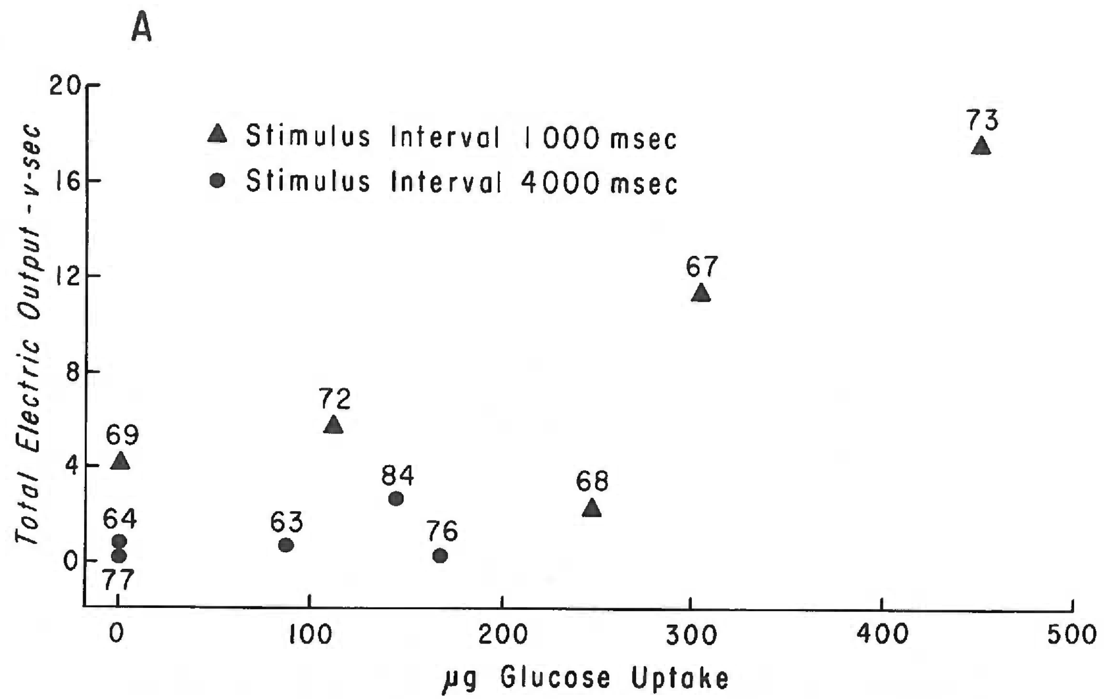
Experiment	Stimulus Interval (msec)	Total Glucose Uptake (µg)	Total Electrical Output (V-sec)	Total Glucose Uptake (µg) / Total Electrical Output (V-sec)	Glucose Uptake mg/g wet wt/hr	Response Amplitude (mV) Start-End
67	1000	306.3	11.55	26.4	2.37	4.0-2.0
68	1000	247.8	2.25	107.7	2.08	0.6-0.5
69	1000	0.0	4.06	—	—	0.7-0.5
72	1000	111.3	5.92	18.9	1.00	1.0-0.85
73	1000	449.0	17.64	25.5	4.22	3.5-2.2
63	4000	86.0	0.74	122.0	0.62	5.0-4.9
64	4000	0.0	0.78	—	—	0.85-0.6
76	4000	168.0	0.16	1050.0	1.22	1.0-0.18
77	4000	0.0	0.10	—	—	0.5-0.18
84	4000	145.5	2.81	51.8	1.29	4.2-4.0
78	0	224.7	—	—	2.00	1.6-1.8
79	0	191.7	—	—	1.80	1.2-1.0
81	0	178.5	—	—	1.63	2.2-2.4
83	0	86.1	—	—	0.79	1.0-0.9
87	0	156.2	—	—	1.41	1.4-1.5

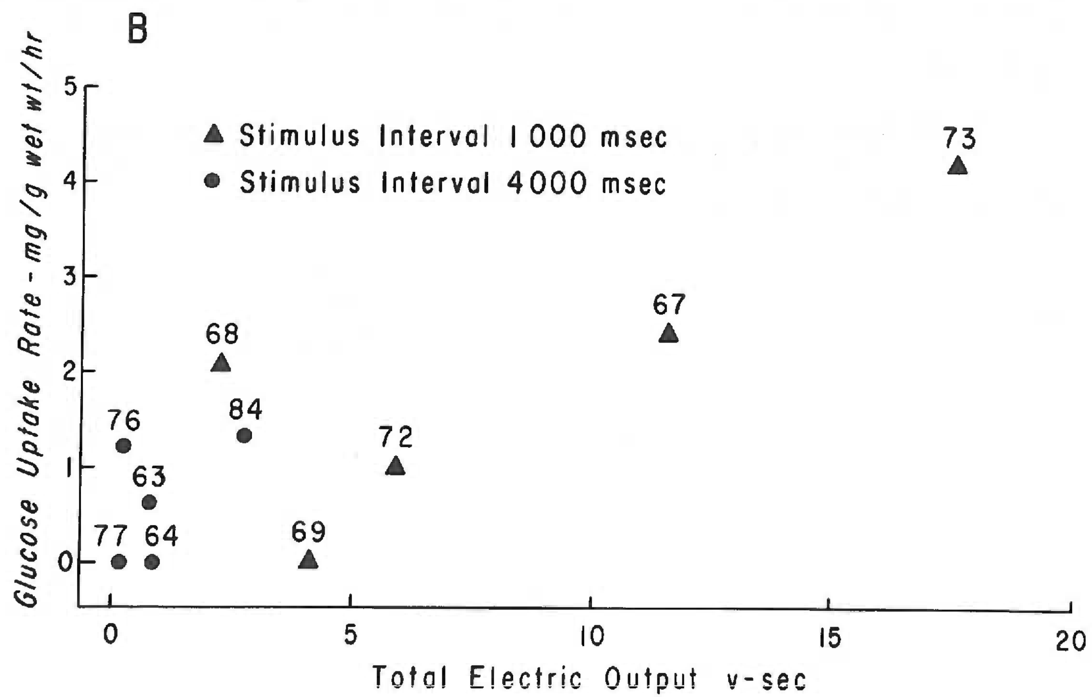
FIGURE 5

Graphic representation of the data presented in Table 3. The numbers indicate the specific experiments.

In A, the total glucose uptake in μg for the experimental period of stimulation is compared to the total electrical output in V-sec.

In B, the glucose uptake rate in mg/gm wet wt/hr is compared to the total electrical output.


A
Total Electric Output - v-sec
μg Glucose Uptake
Stimulus Interval 1000 msec
Stimulus Interval 4000 msec
20
16
12
8
4
0
0
100
200
300
400
500
73
67
72
69
84
68
64
63
76
77


B
Glucose Uptake Rate - mg/g wet wt/hr
Total Electric Output v-sec
Stimulus Interval 1000 msec
Stimulus Interval 4000 msec
5
4
3
2
1
0
0
5
10
15
20
73
67
68
84
76
72
63
77
64
69

total electric output were usually slightly increased. The wide range of variability observed in the individual preparations within each group could be explained by the limitations of the methods of measuring these parameters discussed previously, by the fact that no correlation does exist, or by a combination of both of these factors.

A possible explanation for the lack of glucose uptake in experiment 69 might be found when the beginning viability of the preparation is considered. The recorded responses had an original amplitude of only 0.7 mV. Similar small initial responses associated with no or small uptakes are found in experiments 64 and 77. This explanation does not account for the production of 4.06 V-sec of activity in experiment 69 (unless one postulates two quite different processes controlling the mechanisms of metabolism and functional activity of the tissue) or the 247.8 µg uptake in experiment 68 where the initial response was only 0.6 mV (Table 3).

It was also considered possible that the pre-existing nutritional status of the frogs used might be influencing the uptake data as it was observed that preparations obtained after the animals had been kept refrigerated without food for about a week had larger uptakes. However, it was discovered that all the frogs used in the experiments had been in refrigerated storage without food for at least six weeks prior to their sacrifice. Therefore, it seems that the data of these experiments were obtained from a group of hibernating frogs. The exact nature or role this factor plays in the metabolism being studied is not known.

Although no statistically significant correlation existed between

the glucose uptake and the induced level of activity, graphs A and B (Fig. 5) seem to indicate some degree of relationship when a threshold level of induced electrical activity was exceeded. (Notice plotted points for experiments 69, 72, 67, and 73.) Further evidence for this correlation is provided by the r value for the experimental group stimulated at a 1000 msec interval. This correlation coefficient ($r = 0.91$) is equivalent to a coefficient of alienation of 0.43.

Experiments with Glucose-Free-Ringers.

In the development of the experimental procedure, before the method of glucose determinations was adequately developed and standardized, several examples of glucose production by the spinal cord preparation were encountered. To determine whether this was an error in the methods of determination and sampling or an actual release of glucose from the cord to the perfusate, experiments were conducted in which the preparation was perfused with a Ringers solution to which no glucose was added. The results of these experiments indicated two things:

1) The production of glucose was not real, as no glucose could be detected in the perfusate following periods of stimulation.
2) The stability or durability of the preparation to the continuous stimulation did not seem to be dependent upon the presence of glucose supplied exogenously by the perfusing medium.

In view of this latter suprising observation, several experiments were run in which the perfusing medium consisted of the basic glucose-free-Ringers solution devoid of any exogenous metabolic substrate. These preparations were stimulated at various frequencies and their "durability" compared to preparations which were perfused with a nutrient Ringers. The criterion of durability used for comparison consisted only of the duration of time the preparation would respond to the various stimulation frequencies used. The results of these experiments are listed below.

1. Experiment 13: July 25.

This preparation was stimulated at a 320 msec interval for 113 min. with the response amplitude decreasing from 1.8-0.4 mV. At this point, 5.0 ml of 100 mg% glucose-Ringers was added to the 20.0 ml volume of recirculating perfusate. This produced a glucose concentration (measured by the glucose-oxidase method) of 15.20 mg%. The response changed character by acquiring more spikes, but continued to decrease in amplitude until 18 min. following the administration of the glucose at which time the preparation failed to respond to further stimulation. The cord was allowed to rest for 20 min. and then was stimulated at a 2000 msec interval with a response amplitude of 1.4 mV. However this response deteriorated rapidly.

2. Experiment 14: July 26.

The experiment consisted of 40 min. of stimulation at a 2000 msec interval (resp. amp decreased from 1.75-1.55 mV) and

210 min. of stimulation at an interval of 320 msec (resp. amp decreased from 1.55-0.4 mV).

3. Experiment 21: Aug 8.

This preparation was stimulated for 120 min. at a stimulus interval of 320 msec with the response amplitude decreasing about 40%.

4. Experiment 22: Aug 9.

This preparation was stimulated for 110 min. at an interval of 320 msec at which time a response was no longer obtainable. The cord was allowed to rest for a 15 min. period, then stimulated at a 320 msec interval. The response returned to approximately 60-70% of its original amplitude without the addition of glucose. However, its durability was greatly reduced. After progressing through an "activity burst" (to be discussed later), following 15 min. of stimulation, the cord became unresponsive to further stimulation. Rest periods did allow the cord to recover its capacity to respond. However, the duration of responsiveness was always reduced and the response was usually characterized by an activity burst.

5. Experiment 57: Nov 13-14.

This preparation was stimulated at an interval of 4000 msec. The initial amplitude consisted of a 6 mV spike discharge with a contained slow wave of about 2 mV. The cord responded to this stimulation for a period of 19 hours and 30 minutes.

6. Experiment 59: Nov 16.

This preparation was stimulated at a 500 msec interval. The response was not notably changed following 60 min. of stimulation. The response decreased to about one-third of its original amplitude after 120 min. of stimulation and was still obtainable after 165 min.

The observed durability of these preparations did not seem different from that witnessed when the perfusate consisted of a nutrient Ringers solution. The amount of variability between the two groups was not inconsistent with that seen within the group which had exogenous glucose available for metabolism, and therefore can be accounted for largely by the biologic variability of the preparations.

Activity Bursts.

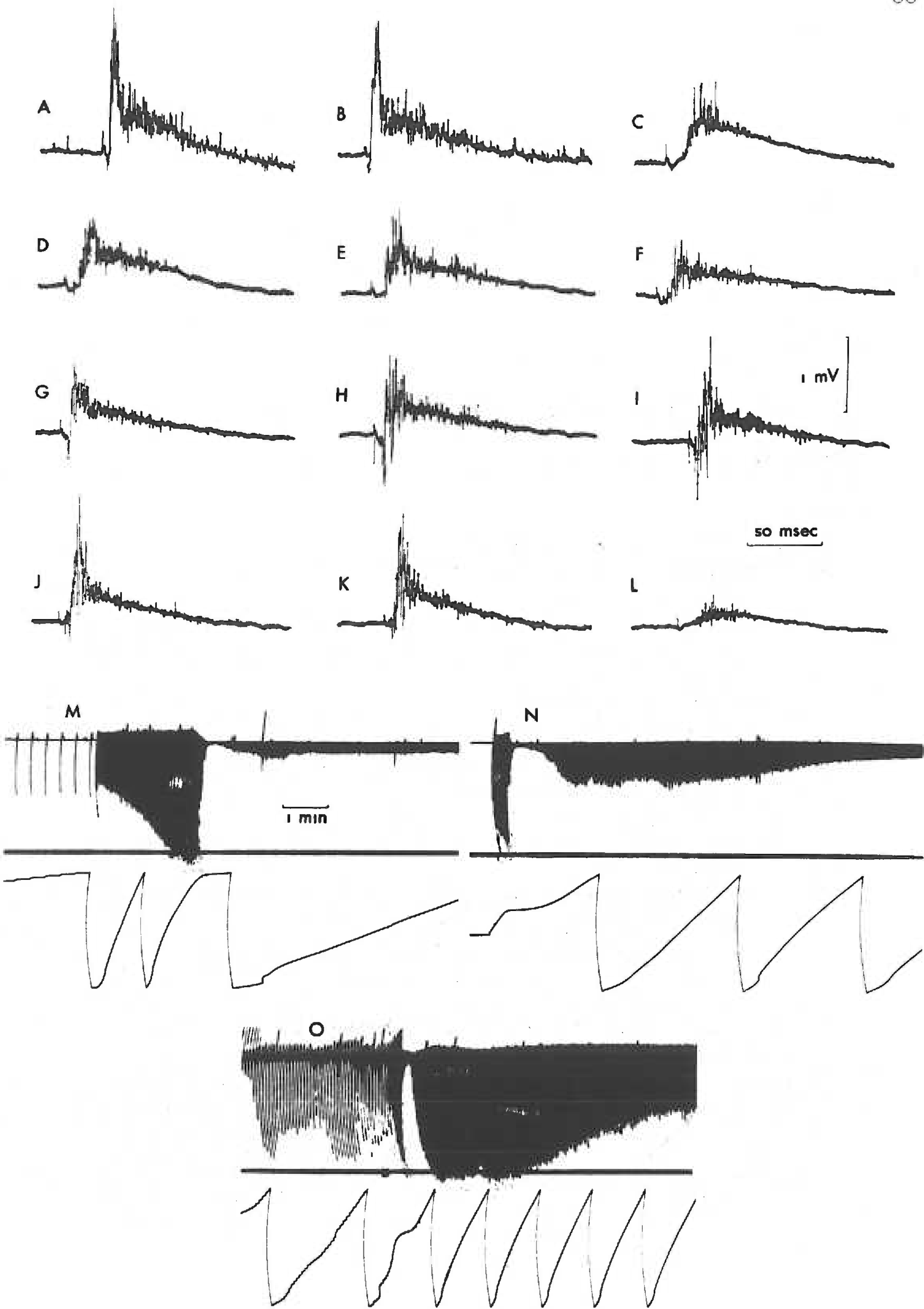
During the course of the experiments, an unusual and unexplained phenomenon was observed in the pattern of the ventral root discharge following extended periods of repetitive stimulation. This event, termed an "activity burst", consisted of a spectacular change in the characteristics of the response. An example of a typical burst is presented in the response series of Fig. 6. Also included in this figure are three examples of Polygraph write out records of such activity bursts.

The first change in the response was a gradual increase in amplitude over a three to five minute period; the characteristics of the individual responses then changed. Because these bursts usually appeared following

FIGURE 6

Examples of ventral root discharge recorded at time intervals during repetitive dorsal root stimulation (A-L). The 3.0 ml perfusate in this experiment contained no glucose.

A) Nature of the response at the beginning of stimulation.
B) Response following 30 min. of stimulation at a stimulus interval of 1300 msec.
C) Response following further stimulation of 60 min. at an interval of 800 msec and 60 min. at an interval of 500 msec.
D) Response following another 40 min. of stimulation at a 500 msec interval.
E) Response after the preparation was allowed to rest 60 min. and then stimulation re-initiated at a 500 msec interval.
F) Response following 15 min. of stimulation at an interval of 500 msec.
G-M) Although these responses were not taken during the same activity burst in this preparation, they are representitive examples of the changes that occur in the response during a typical burst. The time interval between responses would be approximately 90 sec. Absent in these records is the spontaneous discharge and waxing and waning usually observed.
M-O) Three polygraph records of bursts from different preparations. The top tracing in each record represents the amplitude of the response and the lower tracing the record of the integrated accumulative electrical output of the preparation. These records picture the irregular waxing and waning of the response amplitude during a burst.


A
B
C
D
E
F
G
H
I
1 mV
J
K
L
50 msec
M
N
1 min
O

periods of stimulation of considerable duration, the response at the beginning of the burst consisted mainly of a ventral root slow wave with minimal spike discharge. This changed abruptly to a response which was predominantly spike discharge with spontaneous discharge frequently appearing between stimuli. Thus, it appeared as if the slow wave component of the discharge increased in amplitude and then burst into spike discharge; hence, the term activity burst. As shown in the polygraph records (M, N, O in Fig. 6), the activity burst would wax and wane two or three times over a four to five minute time interval with the amplitude of the spike discharge at times increasing as much as 300%. The response characteristics would then return to those of the pre-burst nature with the amplitude always less than that at the start of the burst (L in Fig. 6). The amount of waxing and waning was not constant.

These bursts were observed during the course of repetitive stimulation. The time of appearance of the activity was dependent upon the frequency of stimulation, occurring earlier with higher than with lower rates of stimulation. They were seen with frequency stimulation intervals ranging from 10,000-200 msec. They were seen both in the presence and absence of glucose in the perfusate.

It was found that these bursts could be elicited by repetitively stimulating the cord for two hours at frequencies of 2-3/sec and then allowing the preparation to rest for 20-40 minutes. Following the rest period, the cord would usually respond with an activity burst within 10 minutes after the re-initiation of stimulation.

Since the characteristics of the stimulus were not altered, it was

felt that the nature of the response change was representive of the stimulus becoming more effective in the production of a propagated discharge. This could be explained by partial depolarization of neurone membranes with a consequent increase in the tissue excitability. Such a depolarization would at times have to reach firing levels to account for the spontaneous discharge.

As noted previously (2, 5, 31) excitation results in increased extracellular K^+ and decreased intramitochondrial K^+. This could account for the depolarized state postulated, but seemingly would not account for the waxing and waning observed during the burst and the subsequent return of the stability of the response following the burst. It is difficult to understand how a progressively increasing extracellular accumulation of K^+ could reach levels that would allow the depolarization necessary to account for the activity seen, and then fluctuate in and out of the cell in the time periods necessary to account for the irregularity observed within the activity burst. This would call for the reactivation of fatigued Na^+ - K^+ pump mechanisms or the activation of new mechanisms for the control of ionic concentrations across the cell membrane.

It is also possible that this phenomenon could be associated with synaptic fatigue. Rosenblueth (50) has described similar phases of activity in repetitively stimulated mammalian sympathetic ganglia, and has attributed them to phases in the fatigue of transmission of nerve impulses by acetylcholine. The number of phases produced in his preparations was also dependent upon the frequency of stimulation. The cord preparation

used for the experiments described has been shown to contain internuncial pathways that utilise acetylcholine transmission mechanisms when stimulated by the dorsal root pathway (65). Therefore, the preparation does possess the necessary anatomic and physiologic constituents for the bursts to be explained in this manner. To check the possibility of this explanation, experiments should be conducted with Nembutal blockage of the internuncial pathways. Observations of activity bursts under these conditions would cast considerable doubt on the synaptic fatigue mechanism.

DISCUSSION

DISCUSSION

During the development of the experimental design and procedure, several problems were encountered. One of these, that of micro-organism contamination and the effects of their metabolism on glucose uptakes, is one that is often overlooked by workers undertaking similar investigations. Accounts of attempts to control or check for possible microbial contaminants are found in relatively few articles concerning work of this nature. Although prior to the realization that microbial contaminants were responsible for the major portion of the glucose uptakes noted, no attempt had been made to work with sterile techniques, care had been taken to insure clean material and working conditions. The point to be made is the ease with which the system became contaminated. The results of similar work should be viewed with this in mind. A typical example is the extensive and classic work of Winterstein, done in the early 1900's, on the glucose metabolism of frog spinal cords (64). Some of his experiments extended as long as two days. The probability that his preparations and solutions became contaminated in these prolonged experiments seems to be quite high.

Another factor that might explain a portion of the variability and discrepency of work comparing the nature of metabolic substrates and pathways during rest and excitation is that of the level of excitation.

In most of the reported work, the only comparison made is between stimulated and nonstimulated preparations. No attempt is made to compare the metabolic rates at various levels of excitation. Control of activity levels induced by electrical stimulation is difficult. Yet, with this method of excitation, one has control of the intensity, duration, and frequency of stimulation. With drug induced convulsions, the only control available is that of dosage. Results of the present work suggest that differences do exist in the metabolism of the spinal cord preparation when the metabolic rates associated with different levels of excitation are compared. It was shown that, in the absence of bacteria, resting spinal cords do take glucose from an exogenous source. Activation of the preparation by repetitive, supramaximal stimulation at a frequency interval of 4000 msec revealed uptake rates which were lower, but not significantly so, than those rates observed with resting preparations. Further increases in the activity of the preparation, obtained by decreasing the stimulus interval to 1000 msec, were manifested by increased rates of glucose uptake when these rates were compared to the rates of preparations stimulated every 4000 msec ($0.10 < P < 0.25$). Experiments conducted with glucose-1-C^{14} confirmed the utilization or metabolism of the exogenously supplied substrate by the preparation's production of labeled CO_2; therefore, the term glucose utilization can be substituted for the term glucose uptake.

The results of the present series of experiments are in accord with

the findings of Geiger and others previously discussed in the introduction (3, 21-27, 57). The data seem to be best interpreted by postulating the existence of more than one substrate source. The preference of the source utilized for substrate metabolism is governed by the adequacy of the source to supply energy necessary to meet tissue demands.

Resting preparations require a basal amount of energy to maintain both the state of resting membrane potentials and tissue structural integrity. This source of energy is derived predominantly from exogenously supplied glucose. Mild degrees of activity, characterized by a supposed decrease in the amount of energy required to maintain polarized membranes, are accompanied by questionable decreases in glucose uptakes; but by increases in O_2 consumption and heat production. The latter two observations indicate increased levels of metabolism. In the face of no increase in glucose uptakes during these periods, one must postulate the utilization of an endogenous substrate to account for the increased rates of oxidation. A discrepancy is evident in the above train of thought. It is difficult to explain increased rates of oxidation if one considers activation or depolarization of conductive tissue to be metabolically passive. It could be conceived that the process of repolarization requires more energy than does simple maintenance of a polarized membrane state. The reasons for the apparent change from an exogenous to an endogenous substrate source with activation remains undetermined.

Further stress imposed upon the preparation, in the form of increases in the frequency of stimulation, is accompanied by a return of glucose uptakes to levels higher than those observed either in resting preparations or in those stimulated at lesser frequencies. This could be the result of the endogenous substrate source not being able to supply the energy requirements. But in view of the durability and quantity of this source found with the preparations which were perfused with a fluid devoid of exogenous substrates, this is unlikely. A more likely explanation would be found in the jeopardy of the depletion of the structural components of the tissue, assumed to be identical with or a portion of the endogenous substrate source. This concept is substantiated by the lack of correlation between total glucose uptakes and total measured electrical outputs found when the cord preparations did not exceed an output threshold of 4-5 V-sec of electrical activity during the experimental period, and the suggested relationship noted between the measured parameters when this threshold was surpassed. Therefore, the tissue under environmental conditions simulating those of an *in situ* nature (i.e. glucose available from an exogenous source) does not draw excessively on endogenous stores to supply energy, but does possess the capacity to maintain itself and to meet increased energy demands from endogenous supply sources. It is fitting that an organ system of an essential nature, as the CNS, should contain such a substantial reservoir.

It is felt that the most conclusive findings of this series of experiments are the presence of a prominent endogenous source of

metabolic substrates within CNS tissue, and the ability of this source to maintain the response of the tissue to repetitive stimulation without observed change when the tissue is deprived of all exogenous substrates.

SUMMARY

SUMMARY

1) An attempt has been made to correlate glucose uptakes in a relatively intact preparation of central nervous system tissue to levels of activity induced in this preparation by repetitive, supramaximal, electrical stimulation.

2) It was found that in the experiments conducted, the effects of the metabolism of micro-organism contaminants on the measured glucose uptakes easily masked any glucose uptake by the tissue preparation. Results of similar work in this field should be interpreted with this in mind, if no attempt has been made to eliminate or control possible contamination.

3) Although not statistically significant, the uptake rates of tissue preparations stimulated every 1000 msec were found to be higher than the uptake rates of preparations not stimulated. Preparations stimulated at a 1000 msec interval also had uptake rates higher than preparations stimulated every 4000 msec with the difference significant at the 15% level. A possible theory of CNS metabolic pathways based on the existence of endogenous and exogenous substrate sources and their relation to induced activity levels is presented as an explanation of these results. Such observations are in accord with the findings of other groups of the predominance

of endogenous non-CHO substrate metabolism during drug induced convulsions and electrical stimulation.

5) Evidence supporting the existence of an endogenous substrate source of considerable magnitude and the ability of the tissue preparation to utilize this source in the absence of exogenous substrates is provided by finding no observable differences in the response or "durability" of the preparation when repetitively stimulated in the presence or absence of glucose in the perfusing medium.

6) An unusual and unexplained phenomenon, termed an "activity burst", was observed in the recorded ventral root discharge characteristics following prolonged periods of stimulation. The appearance of this event was noted to be frequency dependent and to possess characteristics of the synaptic fatigue described by Rosenblueth (50).

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APPENDIX A

PREPARATION AND STORAGE OF PERFUSING SOLUTIONS

The nutrient solution used for perfusion of the tissue preparations was that described by Brockhart (14). This consisted of a solution which in ionic composition is similar to frog plasma. The ionic concentration in millimoles per liter is: NaCl 72.6, KCl 3.08, $NaHCO_3$ 34.85, $CaCl_2$ 1.54, and a phosphate buffer to pH 7.4, when equilibrated with a gas mixture of 95% oxygen and 5% carbon dioxide.

Stock solutions of the various components were made as indicated in Table 1.

	F.W.	g Reagent / liter H_2O	% Reagent by weight	Molarity
NaCl	58.45	295.5	25.95	4.4
$NaHCO_3$	84.01	70.0	7.0	0.83
KCl	74.56	11.4	1.14	0.15
$CaCl_2$	147.03	15.8	1.58	0.11
Na_2HPO_4	141.98	25.0	2.5	0.18
NaH_2PO_4	138.01	0.54	5.4	0.39

Table 1: Composition of reagent stock solutions used in preparation of perfusion media. These solutions were stored at 8°C.

From these solutions, two different perfusing solutions were made for the experiments:

1) A dissecting glucose-free Ringers.
2) A glucose-free Ringers solution for experimental perfusion from which the various concentrations of glucose-Ringers were prepared.

The composition of these was identical, only the volume of each prepared was different. Two liters of the first and one liter of the second

were mixed as outlined below:

1) 28.0 ml of the $CaCl_2$ stock was diluted to a volume of 500 ml.
2) The remaining components were mixed and diluted to a total volume of 1500 ml. The amount of the individual component is indicated in Table 2.

Compound	ml of Stock Reagent
NaCl	33.0
$NaHCO_3$	84.0
Na_2HPO_4	20.0
NaH_2PO_4	2.6
KCl	41.0

Table 2: Number of ml of stock solution in 1500 ml of Ringers solution.

3) The 1500 ml solution was then oxygenated with the 95% O_2 - 5% CO_2 gas mixture for 10-20 min before the addition of the $CaCl_2$ solution to prevent the precipitation of insoluble calcium compounds.
4) This 2000 ml solution was then used for the glucose-free dissecting Ringers.
5) In a similar manner, a second solution of 1000 ml was prepared by halving the above amounts.

It was found that the dissecting Ringers could be used for several experiments, and therefore, it was replaced only once or twice a week. After this, it became cloudy due to the accumulation of tissue particles and blood from the dissection procedure.

The second solution was a stock solution from which the nutrient

solutions used for perfusion during the experiments were prepared. Both solutions were kept in refrigerated storage between experiments. In the preparation of the nutrient perfusate, anhydrous dextrose was weighed on a gravimetric scale to the nearest 0.1 mg. These weighed portions were then transferred to 100 ml volumetric flasks and dissolved in the glucose-free Ringers solution. After approximately 50 ml of Ringers had been added to the flask, 0.5 ml of a prepared antibiotic solution containing 2000 units of penicillin and 1000 µg of chloromycetin per ml was injected with a 1.0 cc tuberculin syringe and the solution thoroughly mixed before the remainder of the ringers solution was added to a total volume of 100.0 ml.

In the experiments in which the spinal cords were perfused with glucose-free Ringers, only the antibiotics were added to the Ringers and diluted to the 100 ml volume.

APPENDIX B

SAMPLING PROCEDURES AND DETAILS OF THE CHARACTERISTICS OF THE GLUCOSE-OXIDASE METHOD

Sampling procedures.

Sample size during the first series of experiments at both 100 and 50 mg% perfusates was 0.5 ml. These samples were drawn from a receptacle at the outflow of the chamber with a 1 cc tuberculin syringe. A separate syringe was used for each sample. The sample was then injected into a sample vial which contained two or five milliters of distilled water diluent respectively depending upon whether the perfusate contained 50 or 100 mg. glucose per 100 ml. The syringe was flushed by withdrawing and injecting the diluted sample two to three times. Sample vials were sealed with parafilm and refrigerated until the time of determination, which was never a period longer than 24 hours. Each sample was taken in duplicate as a safety check on concentration determinations.

In a later group of experiments, the volume of the perfusate was reduced to 3.0 ml and was introduced into the experimental chamber from a 10.00 ml volumetric burette. In this series only two samples were taken during each experiment. The chamber was completely drained at the end of the two hour experiment with a 5.0 ml syringe and this fluid, usually 2.6 - 2.8 ml, was used as the end sample. The starting sample was obtained from the remainder of the perfusate in the burette. From both of these samples, two 1.0 ml aliquots were drawn with separate 1.0 ml volumetric pipettes and diluted with 10.0 ml distilled water or a dilution factor of 11. Therefore, with a starting glucose concentration of 100 mg%, the sample to be tested was of 9.09 mg%.

Technique.

Optically matched sets of test tubes were used for all determinations.

These tubes were checked using a 2-5% $CuSO_4$ solution, reading each tube in four quadrants at 510 mμ, and then marking quadrants which did not vary more than 0.003 OD units.

1) After each test tube had been dried in an oven and wiped clean, 4.0 ml of Dades Glucose Oxidase Reagent was introduced with a volumetric pipette.
2) At 60 sec intervals, 1.0 ml of the diluted sample to be tested, or in the case of the blank 1.0 ml of distilled water, was added to the reagent with 1.0 ml volumetric pipettes. Separate pipettes were used for each sample. The time required for this addition was 15-20 seconds. Then for 10-15 seconds, the mixture was stirred with a glass stirring rod to insure a thorough mixing. This allowed 20-30 seconds to pipette the next sample before the time was reached when it was to be added to the reagent.
3) The mixed solutions of reagent and sample were allowed to incubate a timed 10-12 minutes at room temperature, depending upon the number of samples in the determination.
4) Ten drops of 2N HCl were then introduced with an eye dropper into each test tube at 60 sec intervals. This lowered the pH of the solution to a level which inactivates the enzyme preparation and halts the glucose oxidation (34). Following each addition, the solution was again stirred with the glass rod which had been previously left in the test tube.
5) After a 10-15 minute interval, the tubes were then rewiped and OD readings obtained at a 410 mμ wave length using a Beckman B

Spectrophotometer set at zero optical density or 100% trans-mittance.

For each set of determinations a blank was run and each sample was made in duplicate. The OD reading of the sample was taken as the average of the two readings. In the original design and series of experiments in which the 25.0 ml volume was used, the samples were taken in duplicate at each time interval.

Influence of Incubation.

Maximum color development is reached in 60 minutes with the color remaining stable for five hours following 45-60 minutes of incubation. It was found that the diluted samples when allowed to incubate 10-12 min-utes at room temperature, developed a color intensity that yielded OD readings in the desired range of 0.090 - 0.250.

Specificity.

Glucose oxidase has been shown to be relatively specific for beta-glucose oxidation (36). If the rate of oxidation of beta-glucose is taken to be 100, then the ratio of oxidation of beta: alpha-glucose was found to be 100:0.63 at 20°. For this reason, manufacturers of the com-mercial enzyme preparation to be used (Dade) suggestethat solutions of glucose freshly prepared should be allowed to stand at least two hours to insure that mutarotation has reached a state of equilibrium before being used as a standard. All eight D-Alodehexoses have been tested with regards to oxidation by glucose oxidase. If the rate of oxidation of beta-glucose is again taken as 100, then the rates of mannose, altrose, and galactose are 0.98, 0.16, and 0.14 respectively. The remainder of

the aldohexoses show no significant oxidation. G-1-PO_4 and G-6-PO_4 do not serve as substrates for the enzyme system (36).

Sensitivity.

The glucose oxidase reagent curves are supposedly linear and reproducible up to 200 mg glucose per 100 ml (Dade). For greater accuracy if values in excess of 200 mg are obtained, the test should be rerun after diluting the sample with distilled water and then multiplying the values obtained by the dilution factor. It was found that samples had to be diluted into a concentration range of 8-15 mg glucose per 100 ml to obtain readings in the mid-range of the optical density scale on the Beckman B Spectrophotometer used for the determinations.

The sensitivity and variability of the method was determined and checked by several means.

The first method consisted of determining the glucose concentrations of mixed solutions. The solutions were prepared by weighing the anhydrous dextrose on a gravimetric balance to the nearest 0.1 mg and mixing to 100 ml in a volumetric flask with the glucose-free Ringers solution. Stock solutions of 110.0 and 100.0 mg% were made in this manner. These were then diluted in the following proportions to produce solutions of concentrations of 12.00, 10.00, 9.09, and 8.00 mg%:

Standard: 10.0 mg%

1.0 ml of 110 mg% with 10.0 ml Ringers

Sample A: 9.09 mg%

1.0 ml of 100 mg% with 10.0 ml Ringers

Sample B: 8.0 mg%

8.0 ml of 100 mg% qs 100 ml with Ringers

Sample C: 12.0 mg%

12.0 ml of 100 mg% qs 100.0 ml with Ringers

Sample D: 10.0 mg%

1.0 ml of 110 mg% with 10.0 ml Ringers

These samples were then used for the glucose determination. The results are presented in Tables 1, 2, and 3. These results indicate that with glucose concentrations in the range of 8.0-12.0 mg%, the method is sensitive to a 2.0 µg/ml concentration difference.

To determine the effect of the sampling procedure on the sensitivity of the method, eight consecutive 0.5 ml samples were taken from the chamber and diluted with 5.0 ml of distilled water. These were then used for glucose determinations. The results of these determinations are shown in Table 4. The range of the calculated diluted sample concentrations was 9.87 - 10.18 mg% or 3.1 µg/ml.

As the sampling technique was changed with the revision of the perfusing portion of the apparatus, the system was again checked for sensitivity and variability. Solutions of 100, 95, and 90 mg% glucose concentration were mixed. One ml aliquots of these were diluted with 10.0 ml distilled water and used for the determinations. Results are shown in Tables 5 and 6. The calculations indicate variability errors of 1.6, 0.6 and 1.9 µg/ml. These ranges were calculated as the deviation of the determined concentration from the weighed and diluted concentration.

An attempt was then made to check the sensitivity of the method by determining the differences between 100 and 98% solutions after being diluted by a factor of 11. The diluted samples here having a concentration difference of only 1.8 µg/ml or 9.09 - 8.91 mg%. Results are shown in

Tables 8 and 9. Using the diluted 100 mg% solution as a standard (9.09 mg%), the deviation of the determined concentration from the weighed concentration was 0.0 and 1.2 μg/ml respectively for the two determinations.

TABLE 1: Glucose concentration determinations on known solutions. Determinations were made on the same day the solutions were mixed. Readings based at sensitivity three with incubation time of 10 min. Exp. 46; Oct 11.

TABLE 2: Glucose concentration determinations on known solutions. Determinations were made on the day after the solutions were mixed. Readings based at sensitivity three with incubation time 10 min. There was no apparent explanation for the wide deviation in the OD readings of Sample C. Exp. 50; Oct 23.

TABLE 1:
Exp. 46; Oct 11

SAMPLE	OD READING*		OD AVE.	CALCULATED mg%
Std Std	219 222	220 222	221	10.00
A A	198 202	197 203	200	9.05
B B	176 180	177 180	178	8.05
C C	263 265	262 264	263	12.00
D D	221 221	221 220	221	10.00

*OD Readings in this and following Tables have been multiplied by 10^3.

TABLE 2:
Exp. 50; Oct 23

SAMPLE	OD READING		OD AVE.	CALCULATED mg%
Std Std	211 213	211 212	212	10.00
A A	192 193	192 192	192	9.06
B B	170 170	170 170	170	8.02
C* C*	259 250	259 250	259 250	12.21 11.80
D D	212 211	212 211	212	10.00

*When the average OD reading of 255 was used, the calculated concentration was 12.02 mg%.

TABLE 3: Glucose concentration determinations on known solutions. Determinations were made on the same solutions as used in Exp. 50. Time interval between mixing solutions and glucose determinations was two days. Reading based at sensitivity three with incubation time 10 min. Exp. 52; Oct 24.

TABLE 4: Glucose concentration determinations on eight consecutive 0.5 ml samples diluted by a factor of 11. Beginning perfusate concentration was 110.0 mg%. Readings based at sensitivity three with incubation time 10 min. The chamber contained no spinal cord. Exp. 54; Oct 29.

TABLE 3:
Exp. 52; Oct 24

SAMPLE	OD READINGS			OD AVE.	CALCULATED mg%
Std*	232	230	231	238	10.00
Std	240	235	238		
A	219	218	218	216	9.07
A	219	214	214		
B	195	194	195	194	8.15
B	195	191	192		
C	289	287	286	288	12.10
C	290	289	290		
D	241	240	240	241	10.11
D	242	242	241		

*Lost 1 drop from 1 ml pipette in the preparation of sample. Therefore this sample was discarded.

TABLE 4:
Exp. 54; Oct 29

SAMPLE	OD READING		CALCULATED mg%	ORIGINAL MEDIUM CONCENTRATION
Std	232	230*	10.00	110.0
Std	237	234*		
A	231	229	9.87	108.6
B	232	230	9.92	109.0
C	239	238	10.18	111.9
D	235	232	10.00	110.0
E	234	232	10.00	110.0
F	232	231	9.96	109.4
G	231	230	9.92	109.0
H	230	230	9.92	109.0

*The average OD reading of 232 was taken as representative of 10.00 mg% and used in the calculations of the sample concentrations.

Range of calculated diluted sample concentrations:

9.87 - 10.18 = 0.31 mg%
= 0.0031 mg/ml
= 3.1 µg/ml

TABLE 5: Glucose concentration determinations on mixed 100, 95, and 90 mg% solutions with a dilution factor of 11. Solutions mixed and concentrations determined on the same day. OD readings at sensitivity three with incubation time 11 min. Sample A & B at each concentration represent separate dilutions of the weighed mixed solution. Exp. 74; Dec 11.

TABLE 6: Glucose concentration determinations on mixed 100 and 95 mg% solutions. The solutions and dilution were the same as in Exp. 74; Dec 11. OD readings at sensitivity three with incubation time 11 min. Exp. 74; Dec 12.

TABLE 7: This represents a comparison of the concentration of the mixed diluted samples and the determined concentration. Figures in parenthesis indicate the concentration of the undiluted sample. The 100 mg% samples were used as standards and the OD readings of their diluted samples considered to be equivalent to 9.09 mg%.

TABLE 5:
Exp. 74; Dec 11

SAMPLE	OD READING		OD AVE.	CALCULATED SAMPLE CONCENTRATION
100 A	188	186		
100 A	188	186	186	9.09
100 B	187	185		
100 B	189	187		
95 A	183	181		
95 A	182	180	180	8.80
95 B	183	181		
95 B	182	180		
90 C	171	169	169	8.26
90 C	171	169		

TABLE 6:
Exp. 74; Dec 12

SAMPLE	OD READING		OD AVE.	CALCULATED SAMPLE CONCENTRATION
100	185	185	185	9.09
100	184	185		
95	174	172	172	8.45
95	172	172		

TABLE 7:

	CONCENTRATION OF MIXED DILUTED SAMPLE (mg%)	CALCULATED CONCENTRATION OF SAMPLE	DEVIATION mg%
Dec 11	8.64 (95 mg%)	8.80	0.16
	8.18 (90 mg%)	8.26	0.06
Dec 12	8.64 (95 mg%)	8.45	0.19

TABLE 8: Glucose determinations on 100 and 98 mg% solutions with a dilution factor of 11. OD readings at sensitivity three with incubation time of 10 min. Exp. 75; Dec 21.

TABLE 9: Glucose determinations on 100 and 98 mg% solutions (same solutions used in Exp. 75; Dec 21). The same dilution factor, sensitivity setting and incubation time as in Exp. 75; Dec 21 were used. Exp. 75; Dec 26.

TABLE 10: This represents a comparison of the concentration of the mixed diluted samples and the determined concentration. Figures in parenthesis indicate the concentration of the undiluted sample. The 100 mg% samples were used as standards and the OD readings of their diluted samples considered to be equivalent to 9.09 mg%.

TABLE 8:
Exp. 75; Dec 21

SAMPLE	OD READING		OD AVERAGE
100	201	199	198
100	199	197	
98	196	194	193
98	194	191	
100	199	197	198
100	200	198	
98	198	197	195
98	195	193	

Average OD for 100 mg% = 198
Average OD for 98 mg% = 194

TABLE 9:
Exp. 75; Dec 26

SAMPLE	OD READING		OD AVERAGE
100	125	124	122
100*	121	120	
98	119	117	117
98	117	117	
100	119	120	120
100	117	119	
98	118	118	116
98	115	114	

*Lost 1 drop from 1cc pipette when adding glucose sample to enzyme solution.
Average OD for 100 mg% (not counting bad sample) = 121
Average OD for 98 mg% = 117

TABLE 10:

CONC. OF MIXED DILUTED SAMPLE (mg%)		CALCULATED CONC. OF SAMPLE	DEVIATION mg%
Dec. 21	8.91	8.91	0.00
Dec. 26	8.91	8.79	0.12