EFFECTS OF HIGH K^+ MEDIA ON LEUCINE INCORPORATION INTO *APLYSIA* NERVOUS TISSUE

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ABSTRACT

To study possible coupling between membrane polarization and protein synthesis, elevated external K^+ levels were used to depolarize the cell membranes in isolated Aplysia californica parieto-visceral ganglia (PVG). The effect of this treatment on the incorporation of labeled leucine into proteins in the ganglion was analyzed on sodium dodecyl sulfate polyacrylamide gels. PVGs were preincubated 3 hours and then incubated 4 hours in either control medium (^{14}C-leucine, 10 mM $[K^+]$) or experimental medium (^{3}H-leucine, 10 + x mM $[K^+]$ with equimolar $[Na^+]$ reduction). These were homogenized together, separated into aqueous soluble and aqueous insoluble fractions, and run on gels.

In the aqueous soluble fraction of the PVG High $[K^+]$ (90-110 mM $[K^+]$) caused relative increases in incorporation in distinct peaks at 50K (K = 1000 daltons) and 40K. The larger peak, at 50K, was studied further.

The relative increase at 50K occurred when ^{14}C-leucine (instead of the usual ^{3}H-leucine) was incorporated in High $[K^+]$. The relative increase at 50K did not occur (1) when $[K^+]$ was raised to only 50 mM; (2) when $[Na^+]$ was reduced by 80 mM, and $tris^+$ (HCl to neutralize)

was substituted instead of K^+; (3) in pleuro-visceral connective (PVC) nerve; and (4) in the aqueous insoluble fraction of the PVG.

The effect of High $[K^+]$ on incorporation into the giant cell (R2) of the PVG was examined by first labeling the PVG in control medium, rinsing it, and then labeling it in experimental medium. High $[K^+]$ in the experimental medium caused a significant relative increase at 50K in whole PVGs, half PVGs, and R2s dissected from the PVG following incubation. The results in R2 were marred by great variability in the control patterns.

Autoradiography of identified cells (R2 and R15) dissected from PVGs labeled with ^{3}H-leucine in normal $[K^+]$ showed that contaminating cells (mostly glia), which always adhere to such dissected cells, generally account for less than 20% of the total incorporated formalin-fixed label. This contamination is large enough so that a glial origin of the High $[K^+]$ effect on incorporation at 50K cannot be positively excluded. However, the presence of this effect in dissected R2s and its absence in PVC nerves, which contain axons, glia, and connective tissue, but no nerve cell bodies, lend support to the notion that the effect is neuronal in origin.

High $[K^+]$ caused a reduction of approximately 50% in total incorporation into both aqueous soluble and

aqueous insoluble proteins of the PVG. Similar decreases of 35% were seen in dissected R2s. [Na^+] reduction (by 80 mM, $tris^+$ substitution) had no significant effect on total incorporation (measured only in the aqueous soluble fraction of the PVG). High [K^+] caused a reduction of approximately 85% in total incorporation into PVC nerve. Autoradiography of the nerve showed that this reduction occurred in both the connective tissue sheath and the axonal-glial region. High [K^+] caused no significant change in non-volatile TCA soluble label in either the ganglion or the nerve.

Other effects of High [K^+] on the PVG: (1) a small (not large enough to have caused the relative increase at 50K) decrease in the relative amount of label in the aqueous soluble, TCA insoluble fraction compared to the aqueous insoluble fraction, and (2) a relative decrease in incorporation in higher molecular weight peptides compared to lower molecular weight peptides in both aqueous soluble and aqueous insoluble fractions.

These results suggest, but do not prove, that High [K^+] caused an increase in the synthesis of a neuronal peptide of approximately 50,000 daltons molecular weight. The possibility that this peptide may be a tubulin subunit is briefly discussed.

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CHAPTER I

INTRODUCTION

About 40% of the dry weight of the mammalian brain is protein (McIlwain & Bachelard, 1971, p. 246). The high rate of synthesis of brain proteins (Gaitonde & Richter, 1955; Lajtha, et al., 1957) indicates their probable functional importance. Protein synthesis, and in particular, the modulation of protein synthesis, have played important roles in speculations about the modification of neural activity and connectivity as the result of electrical activity in the brain (Schmitt, 1964; Hyden, 1970; Rapoport & Daginawala, 1970; Ungar, G., 1968). As will be discussed below, changes in the metabolism, amounts, and/or properties of particular proteins in the brain accompany changes in behavior or neuronal activity. Understanding the control and modification of the protein synthesis in the brain is therefore, probably important to understanding how the brain functions, and it was with this thought in mind that I chose as my thesis project a study of some aspects of the modification of protein synthesis in neural tissues.

In this introduction, I discuss, first of all, previous studies on protein synthesis in neural tissues. The method I used to modify protein synthesis was to raise external potassium levels, and the rationale for this

procedure is discussed in the following section. Finally, the tissue I used was the parieto-visceral ganglion of Aplysia californica, and the advantages of using this preparation are discussed in the final section of this introduction.

PREVIOUS STUDIES ON PROTEIN SYNTHESIS IN NERVOUS TISSUE

In vivo experiments

Protein metabolism of brain has been studied in animals placed in novel situations. These novel situations include sensory stimulation, forced motor activity, electro-convulsive shock, induced convulsions, and learning situations.

Situations other than learning[1] situations. Talwar and associates have investigated the effect of exposure of animals to light (as opposed to constant dark) on protein synthesis in occipital cortex. Talwar, et al. (1966) observed an increase in incorporation of ^{14}C labeled glucose or valine into proteins of occipital cortex in adult rabbits exposed to 30 minutes or 2 hours of 7/sec flickering light. A similar result was obtained with Rhesus monkeys using ^{3}H-lysine as the label (Singh & Talwar, 1967). In both rabbits

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[1] "Other than learning" in the sense that the animals in these experiments are not required to learn a particular task. The animal may, of course, be learning something anyway.

and monkeys, the effect is seen in both soluble and particulate fractions. Some of the proteins which have increased labeling have similar chromatographic characteristics to the S-100 brain specific protein (Moore & Perez, 1968), known to be present in monkey occipital cortex (Singh & Talwar, 1969).

Wegener (1970) found that unilateral eye removal in frogs subsequently (10 days later) exposed to light produced relative increases in ^{3}H-histidine incorporation in tectum contralateral to the remaining eye. Similarly, Bondy & Margolis (1970) unilaterally enucleated chicks' eyes and found that ^{3}H-leucine incorporation into protein was higher in optic lobes and whole cerebral hemisphere contralateral to the seeing eye one day or 17 days after eye removal. These results obtained using intra-animal controls, are similar in direction to Talwar's. This suggests that the effects of light seen by Talwar were not due to different degrees of stress involved with exposure to light or dark.

Rose (1967) found that rats first exposed to light at age day 50 increased incorporation of ^{3}H-lysine into visual cortex, but only during the first three hours of light exposure. Subsequently, the animals remaining in light incorporated relatively less than animals kept in the dark. Bateson et al. (1969) found that a flashing light produced an increase in incorporation of ^{3}H-lysine into the roof of the forebrain of chicks.

Increased motor activity has also been shown to be accompanied by changes in protein synthesis. Rats made to run at about 25 ft/min in a running wheel showed an increase in labeling of neurons with ^{3}H-leucine during the exercise (Altman, 1963). This increase was shown in all areas of the brain and in spinal motoneurons. A similar result was obtained in motor cortex by Rose (1967). Such results have also been obtained with ^{35}S-methionine in a swimming task in rats by Krawczynski (1961). In contrast, Jakoubek & Gutmann (1968) found a decrease in incorporation of ^{35}S-methionine into spinal motoneurons of rats during swimming. Tiplady (1972) found a decrease in incorporation of ^{3}H-lysine into cerebral cortex of rats during running at about 8 ft/min. The reasons for these discrepancies are not known; no two of the experiments on motor activity are alike in all (or even all but one) respects.

In the experiments discussed above, the levels of sensory stimulation or motor activity were not excessive, although the treatment of the animals or the means of obtaining the experimental "activity" may be regarded as unusual. The function of the observed changes in protein synthesis is unknown though one may guess that they represent adaptive responses to changes in nervous activity.

Protein synthesis has also been observed in situations which may be regarded as essentially non-physiological, such as during and after convulsions and during spreading

depression. Electroconvulsive shock (ECS) is known to have many long term effects on behavior and brain biochemistry (briefly reviewed in Dunn, et al., 1971). ECS has acute effects as well: using either ^{3}H-leucine or ^{14}C-glucose as precursor (and expressing incorporation relative to label in amino acids), Dunn, et al. (Dunn & Guiditta, 1971; Dunn, Guiditta & Pagliuca, 1971) showed a 20 to 70 percent inhibition of protein synthesis in mouse brain during the first five minutes after electroschock. A significant but smaller decrease in synthesis of protein was present one to 24 hours after ECS as well.

Ruscak (1964) showed that spreading cortical depression (SCD) decreased ^{35}S-methionine incorporation into proteins of brains of anesthetized rats during the first 30 minutes or first 90 minutes after SCD was elicited. Bennett & Edelman (1969) similarly showed a decrease in incorporation of ^{3}H-leucine into cortical proteins of unanesthetized, freely moving rats during spreading depression. Fractionation of proteins by centrifugation and disc polyacrylamide gel electrophoresis did not reveal differential effects on specific proteins. In both of these studies, SCD was elicited unilaterally and the comparison was made between the two hemispheres; decreases were approximately 15 to 25 percent.

The one type of peptide synthesis of the brain for which we have at least a partial idea of the function of the

peptide is in the synthesis of peptide neurosecretions. Among these peptide neurosecretions are the vasopressins, the oxytocins, and the various hypothalamic releasing factors. With regard to their synthesis, most information presently available is on vasopressin. Slices of hypothalamus taken from guinea pigs exposed to four days of water deprivation (known to cause release of vasopressin) incorporated two to four times as much ^{35}S-cysteine or ^{3}H-tyrosine into vasopressin as did slices from control animals. Incorporation into total protein of the tissue was not changed (Takabatake & Sachs, 1964). Similar treatment (2% NaCl instead of drinking water for three days) is known to increase the firing rates of hypothalamic neurons which send processes to the neural lobe (presumed to contain oxytocin or vasopressin) but not other hypothalamic neurons (Dyball & Pountney, 1973). Sachs, et al. (1967) looked for but could not find any immediate effect of hemorrhage (also known to cause vasopressin release) on vasopressin synthesis either <u>in vivo</u> or <u>in vitro</u> on slices of hypothalamus taken after bleeding.

<u>Learning situations</u>. Hyden's laboratory has studied changes in rats associated with learning to use the non-preferred paw. They found changes in the relative amount of particular electrophoretic fractions of pyramidal cells which were dissected freehand from the CA3 region of the hippocampus (Hyden & Lange, 1970a,b). A fast running band

stained by amido schwarz appeared in tissue from trained animals. The band has been identified as the S-100 protein (Moore & Perez, 1968) by the following criteria: the band dissolved in saturated ammonium sulfate, gave a positive immunological reaction with antiserum against the S-100 protein and ran on the gel near where S-100 would be expected (Hyden & Lange, 1970b). The appearance of this band of S-100 may not necessarily mean that the new S-100 has been synthesized, since there is evidence that the S-100 of controls runs slower on the gels. The new band may just be a different conformational species (Haljamae & Lange, 1972). The claim was made that the sum of the usual S-100 band and the new one is increased in trained animals; however, the increase is only 10% and may not be significant.

Using the same training paradigm, they also found an increase in incorporation of intra-ventricularly injected ^{3}H-leucine into proteins of pyramidal cells of the hippocampus (Hyden & Lange, 1968). The claim was made that "protein synthesis of two fast-moving fractions (in polyacrylamide gel electrophoresis) was significantly higher bilaterally in the hippocampus of trained rats (than in controls)." Similar results were obtained for incorporation into total unseparated proteins of these cells. Yanagihara and Hyden (1971) studied the incorporation of ^{3}H-leucine into proteins of whole regions (i.e., CA1, CA3, and CA4) of the hippocampus, and made a similar claim for the total

protein of the CA3 region. A more recent paper (Hyden & Lange, 1972), using intraperitoneal instead of intraventricular injection, showed decreases in incorporation into hippocampal proteins.

One of the problems with Hyden's incorporation experiments, as well as many of the papers discussed in the previous section, is that they make use of what may be a spurious correction factor derived from the measurements of trichloracetic acid (TCA) soluble label. In Hyden's experiments, the difficulties with this correction factor are the following: (1) The data demonstrating a linear relation between specific activity of proteins and TCA soluble label (Hyden & Lange, 1968) showed great scatter and, indeed, may not be linear at high levels of TCA soluble labels; (2) The TCA soluble material contains metabolic products of leucine as well as leucine itself (Banker & Cotman, 1971); and (3) although the experiments in Hyden & Lange (1968) and Yanagihara & Hyden (1971) are essentially identical (the only difference is the anesthetic used), the TCA soluble label in the CA3 region of the trained animals fell drastically in the former paper, but did not change at all in the latter. This discrepancy was not discussed by the authors. Some of these criticisms have been suggested previously (Bowman & Harding, 1969).[1] The experiments of Hyden and co-

[1]There does not seem to be a completely satisfactory

workers on leucine incorporation into brain proteins, while interesting, remain essentially uninterpretable.

Kerkut's group has done in vivo studies of learning in the cockroach and garden snail. Headless cockroaches suspended over a saline bath, from which they would receive a shock if they lowered their leg, learned to keep their leg up (Horridge, 1962). Kerkut, et al. (1972) injected ^{3}H-leucine into animals to be trained one hour before conditioning. ^{14}C-leucine was injected into controls (yoked controls which received unavoidable shocks whenever the trained animals were shocked). Analysis on disc polyacrylamide gels of the aqueous soluble fraction of the metathoracic ganglia of the control and experimental animals showed relative increases in the trained animals in incorporation into one, and possibly three, fractions observed on these gels.

The garden snail experiments (Emson, et al., 1971) were very similar to the cockroach experiments: the snail was shocked on the tentacle whenever the tentacle was extended and soon learned to keep its tentacle retracted.

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way of dealing with this problem of precursor specific activity. Among the solutions suggested or used are (1) use of carboxyl labeled amino acids, particularly valine (Banker & Cotman, 1971), (2) use of labeled glucose, accompanied by measurement of specific activity of amino acids, or (3) comparison of incorporation into different proteins to find relative differences of synthetic rate of proteins.

A yoked control, receiving shocks on the tentacle whenever the trained animal was shocked, was used as in the cockroach experiments. Analysis of ^{3}H-and ^{14}C-leucine labeled proteins of the brains of the trained and control animals showed relative increases in the trained brains into at least two fractions observed on disc polyacrylamide gels.

This would indicate that learning in the cockroach and garden snail is accompanied by a relative increase in synthesis of particular proteins, unless the effects reported are due to (1) changes in the specific activity of the pools from which these proteins are synthesized, or (2) a difference in the pattern of proteins synthesized with ^{14}C and ^{3}H labeled precursors. Kerkut did not report on controls to check these possibilities.

Ungar has probably produced the most convincing evidence that particular peptides are present in the brains of trained animals which are not present (or are of much lower concentration) in naive animals. Animals trained to avoid the dark have a substance present in their brain which, when injected into naive animals, caused dark avoidance behavior. The substance was sensitive to proteolytic enzymes, and upon purification, appeared to be a pentadecapeptide. The purification technique has now been refined to the point that a chromatographic spot which can be dansylated can be found in brain tissue from trained animals while it is absent from brain tissue from naive

animals subjected to the identical purification procedures (Ungar, et al., 1972).

Ungar and co-workers have worked with a number of other trained behaviors, including airpuff habituation, loud sound habituation, and stepdown avoidance. Again, substances are present which are sensitive to proteolytic enzymes and which induce homologous behaviors in naive recipient animals (Ungar, 1971). A few of these have been purified enough to identify their constituent amino acids (Ungar, 1972). Behavior induction by means of brain extracts of trained animals has been observed in many other laboratories (Ungar, 1971), suggesting that this is a real, though sometimes difficult to observe (e.g., Goldstein, et al., 1971), phenomenon. No other laboratory has carried the chemical analysis of these substances as far as Ungar's.

Several other experiments on changes in protein metabolism, associated with learning are reviewed in Glassman (1969) and Uphouse, et al., (1973).

In vitro experiments

Most in vitro experiments have utilized brain slices from vertebrate brains, although recently a number of experiments using the superior cervical ganglion and invertebrate nervous systems have been done. The preparation of brain slices is explained in great detail by McIlwain & Rodnight (1962), and many recent studies using this preparation make

reference to this work. Generally, slices about 0.3 mm thick are cut from the surface of the brain(usually guinea pig or rat). Slices are placed in an oxygenated physiological saline. They maintain ion concentrations much like those found *in vivo* and exhibit resting potentials as large as -90 mV. They can be electrically stimulated or subjected to addition of drugs or changes of ionic constituents in the incubation fluid. Under normal conditions, incorporation of ^{14}C-valine into proteins of such slices is linear for at least four hours (Orrego & Lipmann, 1967).

Orrego and Lipmann (1967) studied the effects of electrical stimulation and acidic amino acids on the incorporation of ^{14}C-valine into brain slice protein. Electrical stimulation of the slice for 15 to 30 minutes caused a decrease of about 40% in incorporation into protein. Orrego and Lipmann did a large number of control experiments, showing (1) electrical stimulation did not change the amino acid uptake of the noninulin space of the brain slice; (2) the result was the same if labeled glucose was used as precursor (after correcting for an increased conversion of glucose to amino acids caused by stimulation); and (3) stimulation after incorporation did not decrease incorporated label. Essentially identical effects were produced by acidic amino acids which were known to have excitatory effects on vertebrate nervous tissue (glutamate, homocysteate, aspartate, and aminoadipate). The magnitude

of the effects of acidic amino acids ranged up to a high of 47% inhibition with homocysteate and approximately paralleled their excitatory effects on neurons (Curtis & Watkins, 1963). Protein synthesis in kidney slices was unaffected by either type of treatment.

Jones and Banks (1970) also observed a similar decrease of incorporation of ^{14}C-valine into brain slice protein after 15 minutes of electrical stimulation. At shorter periods of stimulation (not used by Orrego and Lipmann) increases in incorporation of up to 85% were observed; however, the increase seemed to be due to an increase in valine uptake early in stimulation (Jones & Banks, 1970).

Jones and McIlwain (1971) tested the effects of increasing $[K^+]$ in the incubation medium on ^{3}H-leucine incorporation. They found that added KCl decreased incorporation into protein. Measurements were made for $[K^+]$ between 3 and 48 mM, and no effort was made to control for changes in osmotic strength. 48 mM $[K^+]$ inhibited ^{3}H-leucine incorporation by as much as 85%.

Folbergrova (1961) examined the incorporation of ^{35}S-methionine into brain slice proteins. High $[K^+]$ medium, produced by adding excess (100 mM) KCl or making an isosmotic substitution for NaCl($[K^+]$ ranging from 0 mM to 130 mM), reduced incorporation into protein by as much as 70%. No measurement of the effect of $[K^+]$ on methionine

uptake is reported. Glutamate decreased methionine incorporation into brain slice protein. Ca^{++}-free medium inhibited incorporation about 40%. Similar results have been obtained by Mase, et al., (1962) using ^{14}C-glycine as precursor. Control experiments on liver slices showed little or no effects of $[K^+]$ or $[Ca^{++}]$ changes.

In order to localize effects on protein synthesis in brain slices to either glia or neurons, Blomstrand and Hamberger (1970) incubated brain slices as usual and then fractionated the tissue into glial and neuronal-enriched fractions. High $[K^+]$ media caused an increase in incorporation at moderate (40 mM) levels but a decrease at 100 mM in both glial and neuronal fractions. In this experiment K^+ was added in excess and osmotic strength was equalized in the control media by addition of sucrose.

Protein synthesis in the superior cervical ganglion (SCG) of the rat has recently been studied by McBride & Klingman (1972). The SCG was stimulated by means of a suction electrode on the pre-ganglionic fibres. With ^{14}C-glucose as precursor, stimulation increased the specific activity of free amino acids in the ganglion over the unstimulated control. Stimulation had no effect on incorporation into proteins; however, if one assumes that the measured amino acid pool is similar to the pool from which the proteins were synthesized, then a decrease of 14-25% in protein synthesis occurred.

Wilson and Berry (1972) studied the effect of synaptic stimulation on the proteins synthesized by a single, identified large cell in the parieto-visceral ganglion of Aplysia californica. Patterns of labeled leucine incorporation observed on SDS polyacrylamide gels did not change. Total incorporation did not change significantly; however, the variability was such that even a 25% change in the mean was insignificant.

In brain slice experiments, electrical stimulation decreased protein synthesis. The same result was obtained with the superior cervical ganglion. The relation of this in vitro change to in vivo observations is not well understood. Jones and Banks (1970), noting that the method of stimulation produced general excitation rather than the complex patterns of electrical activity normally observed in cortex, suggested that the conditions used might be likened to those occurring in vivo during convulsive activity. The decreases in protein synthesis actually observed by Dunn and Guiditta (1971) immediately after electroshock support this idea.

An unanswered question in these in vitro experiments was whether all proteins in nervous tissue are affected equally by stimulation. This is the question I sought to answer. This question seemed of interest in the light of possible effects on specific proteins which some in vivo experiments had shown. The method of stimulation I chose

was that of raising external K^+ levels, the rationale for which is discussed in the next section.

RATIONALE FOR USING HIGH K^+ MEDIA TO STIMULATE NERVOUS TISSUE

In this section, the following topics will be discussed:

(1) the dependence of the resting membrane potential on external $[K^+]$,

(2) the efflux of intracellular K^+ caused by electrical activity,

(3) similarities between electrical stimulation and raising external $[K^+]$, and

(4) possible physiological roles of increased levels of external $[K^+]$.

The idea to be developed is that raising external $[K^+]$ is a convenient method of depolarizing brain cells, has effects similar to electrical stimulation, and may resemble, in a gross manner, a physiological process taking place in the nervous system.

The dependence of the resting membrane potential on external $[K^+]$

The membrane potential of nerve, muscle, and glial cells is dependent upon the distribution of K^+ across the membrane. The potential is approximately that of a K^+

electrode, the behavior of which is described by the Nernst equation:

$$E_{K^+} = \frac{RT}{F} \ln \frac{[K^+]_{\text{outside}}}{[K^+]_{\text{inside}}}$$

According to this equation, the membrane potential should become approximately 58 mV less negative if the external potassium concentration is raised ten-fold. This behavior has been verified in many systems, notably in molluscan neurons (Gorman and Marmor, 1970; Kerkut & Meech, 1967; Carpenter & Alving, 1968), squid giant axon (Curtis & Cole, 1942), vertebrate nerve (Huxley & Stampfli, 1951), vertebrate neurons, (Blackman, et al., 1963) vertebrate and invertebrate glia (Kuffler & Nicholls, 1966), and vertebrate muscle fibres (Hodgkin & Horowicz, 1959).

The efflux of intracellular K^+ caused by electrical activity

Normally, the internal K^+ concentration of nerve cells and glial cells is about 15 times higher than the external K^+ level (External $[K^+]$ is about 2.5-5 mM for terrestrial and aquatic forms and about 10 mM for marine forms.) During an action potential the permeability of the neuronal membrane to sodium and subsequently to potassium momentarily increases. Potassium leaks out of the cell; sodium, normally low inside the cell and high outside, leaks in.

Squid giant axon loses 3 to 4 x 10^{-12} mole K^+/cm^2 per impulse (Hinke, 1961, and others: several references given in Baylor & Nicholls, 1969). Brain slices also decrease their K^+ content in response to electric stimulation. Thus, Jones and McIlwain (1971) observed a 30% reduction in K^+ content following 15 minutes of 50 Hz stimulation. Similar results have been obtained by Jones & Banks (1970) and others (reviewed in McIlwain & Bachelard, 1971).

This amount of K^+ entering the extracellular space during electrical activity is non-trivial, and, indeed, can cause sizable--and measureable--changes in the normally low extracellular $[K^+]$. Frankenhaeuser and Hodgkin (1956) found in squid giant axon that repetitive firing of the nerve at 50 per sec caused a decrease in the action potential undershoot. (The undershoot is mainly dependent on the K^+ distribution across the membrane, and varies in a nearly Nernst-like manner with external $[K^+]$). They attributed this decrease to an increase of $[K^+]$ adjacent to the membrane of approximately 1.6 mM/impulse. Baylor and Nicholls (1969), similarly using the undershoot of the action potential, observed in the leech an increase in extracellular $[K^+]$ adjacent to a neuron amounting to .8 mM/spike. Neher and Lux (1973) used ion-specific K^+ electrodes to show that extracellular K^+ adjacent to snail neuron cell bodies increased following action potentials. They shows a 1 mM

increase of $[K^+]$ in the course of 7 action potentials given at the rate of 3 per second.

Nicholls and his colleagues have exploited the fact that the glial cell resting potential behaves as a very good K^+ electrode to demonstrate the build-up in extracellular $[K^+]$ during repetitive stimulation. Using a maximal 10/sec stimulation of the optic nerve of the amphibian Necturus, Orkand, Nicholls, & Kuffler (1966) observed depolarizations in glial cells of the nerve corresponding to an increase of extracellular $[K^+]$ of 15 to 25 mM. In the leech, Baylor and Nicholls (1969) recorded from a ganglionic glial cell while stimulating the posterior root maximally at 15 per second. Depolarizations in the glial cell corresponding to an average increase of $[K^+]$ of 10 mM were seen, but, as the authors state, "This figure must represent a lower limit to the build-up which accumulated near some regions of the glial membrane, since portions of the cell and others to which it is electrically coupled face extracellular space in which the K^+ build-up would inevitably be smaller." Karahashi and Goldring (1966) have also observed in mammalian cortex slow depolarizations of "idle cells," presumed to be glia (Castellucci & Goldring, 1970), in response to direct stimulation of the cortex or indirect stimulation via stimuli to thalamic nuclei. These observed depolarizations would correspond to increases of extracellular $[K^+]$ of between 5 and 20 mM (Prince, 1971).

The rates of stimulation used in the above experiments are not at all unusual for neurons. The glial depolarizations observed by Orkand, et al. (1966) in the optic nerve of Necturus could be observed in response to light shone in the eye. In Baylor and Nicholls' (1969) experiment on the leech, measurements were made on a "pressure neuron" using pressure on the skin as the stimulus. Frequencies of over 60/sec are easily produced and lead to an increase in $[K^+]$ adjacent to the neuron calculated at 4 mM or more in less than a second.

Similarities between electrical stimulation and raising external $[K^+]$

In in vitro experiments on brains and ganglia, both high $[K^+]$ and electrical stimulation inhibit protein synthesis (see previous section), increase respiration and glycolysis (Orrego & Lipmann, 1967; Larrabee, 1958; Mase, et al., 1962; Ashford & Dixon, 1935), decrease high energy phosphate reserves (Heald, 1960), increase the rate of cyclic adenosine monophosphate (cAMP) synthesis (Kakiuchi, et al., 1969; Shimizu & Daly, 1972; Cedar, et al., 1972), and increase transmitter and neurohormone release (McIlwain & Bachelard, 1971; Arch, 1972) of nervous tissue. Thus, many of the effects of raising external potassium are similar to those produced by electrical stimulation.

Some of these effects are undoubtedly due simply to

the depolarization of neurons that both treatments cause. Thus, this mechanism explains the release of transmitters and neurohormones. Such a mechanism has also been suggested in the explanation of the increase of cyclic AMP synthesis (Shimizu & Daly, 1972).

In view of the large efflux of K^+ into the extracellular space during electrical stimulation, it seems possible that the common link between electrical stimulation and raising external $[K^+]$ is simply that both treatments increase $[K^+]$ outside neurons and glial cells. In addition, such increases of external $[K^+]$ may have other direct effects on neurons and glial cells.

The depolarization of glial cells brought about by increases of external $[K^+]$ may be responsible for some of the effects common to electrical stimulation and high $[K^+]$ treatments. To my knowledge, glial depolarization has not been suggested in discussions of these phenomena.[1] The experiments on cyclic AMP which suggested the involvement of neuronal depolarization utilized depolarizing agents (K^+, veratridine, ouabain, and batrachotoxin) which may affect glial membranes as well. Therefore, a mechanism of this type is not ruled out.

Other effects of increased external $[K^+]$ have been

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[1] Kuffler & Nicholls, 1966, have, however, speculated that this type of mechanism may be involved in the possible trophic function of glia. See p. 24.

invoked to explain the action of electrical stimulation on protein synthesis, respiration, and high energy phosphates. Heald (1960, p. 119) suggested that the influx of Na^+ and the efflux of K^+ caused by electrical activity results in an increased energy usage by ion pumps in the membrane. These ion pumps, which act to restore the initial distribution of ions across the membrane, are known to utilize high energy phosphate compounds. This would, therefore, decrease the store of high-energy phosphates and divert these compounds from less urgent functions, such as protein synthesis. The increase in respiration would be a compensatory mechanism to keep up with the increased demands for high energy phosphates. Similarly, Heald (1960, p. 133) suggested that, raising external $[K^+]$ would also increase the activity of cellular ion pumps, thereby increasing energy expenditure, and producing the same result as electrical stimulation.[1]

The point to be noted from this discussion is that electrical activity, a normal component of nervous function, and high $[K^+]$ treatment, the manipulation used for most

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[1]This explanation is undoubtedly overly simplified. Jones and McIlwain (1971) have shown that neither the kinetics nor the thresholds (frequency for electrical stimulation, concentration for potassium) for these effects were exactly correlated with the drop in phosphocreatine in the tissue. Since ion pumps play a central role in the suggested mechanism, I find it incredible that I cannot locate an article in which the effect of ouabain, an ion pump inhibitor, has been tested for its effect on these phenomena.

of the experiments in this thesis, have many features in common.

Caveat. It must be pointed out that the levels of K^+ used in most of the experiments in this thesis and in most brain slice work are probably higher than those normally encountered in the nervous system, even under the most extreme conditions of electrical stimulation. Levels of K^+ which depolarize neurons much above the threshold potential cause them to become electrically inexcitable (Curtis & Cole, 1942). When external $[K^+]$ is high enough to block conduction, the efflux of K^+ accompanying electrical activity must cease. Levels of external K^+ above this point must, therefore be considered non-physiological. Electrical inexcitability, which accompanies higher levels of K^+, probably does not normally occur with the long time scale used in most high $[K^+]$ experiments. Biochemical changes, at these higher "non-physiological" K^+ concentrations may be extreme manifestations of phenomena taking place at lower K^+ levels. One must be aware, however, that they may be completely new phenomena which take place only at K^+ concentrations above the level needed to block conduction.

Possible physiological roles of increased extracellular $[K^+]$

As has already been discussed in the section on K^+ efflux during electrical activity, increases of

extracellular $[K^+]$ can occur during normal activity of the nervous system. Experiments with high $[K^+]$ therefore take on added significance if these increases of extracellular $[K^+]$ have roles in the function of the nervous system.

Specific suggestions for such roles have been made by several authors. In considering the possible trophic function of glia, Kuffler and Nicholls (1966) suggested that the K^+ mediated depolarization of glia "may be a mechanism by which neurons provoke glial cells to supply metabolites." John (1966) speculated that extracellular K^+ may play a role in learning and retention. According to his hypothesis, changes in glial and neuronal K^+ brought about by electrical activity could modulate protein synthesis of these cells in such a way that the neurons involved would be more likely to respond to particular temporal patterns of input. Izquierdo and associates have provided evidence that K^+ accumulation and release in hippocampus is associated with learning. Rats of low inborn learning ability released less potassium into extracellular space of the hippocampus in response to stimulation of the fornix than did rats of intermediate learning ability (Izquierdo & Orshinger, 1972). It was also shown that in a normal population of rats, performance in a shuttlebox task was correlated with the ability of the hippocampus to release K^+, when stimulated (Izquierdo, et al., 1972). These effects are also correlated with

responses of RNA synthesis in the hippocampus to topically applied K^+. Accordingly, they have speculated that learning involves the release of K^+, which triggers biochemical events initiated by RNA synthesis.

ADVANTAGES OF USING THE PARIETO-VISCERAL GANGLION OF *APLYSIA CALIFORNICA* FOR BIOCHEMICAL STUDIES

The preparation I used is the parieto-visceral ganglion of *Aplysia californica*. This preparation has a number of advantages over the brain slice preparations used in most previous studies on protein synthesis in neural tissues.

(1) The preparation can be maintained in a functionally active state *in vitro* for several days in a simple sea water medium. It can be maintained *in vitro* for more than six weeks in a modified Eagle's culture medium (Strumwasser & Bahr, 1966; Strumwasser, 1967).

(2) The neurons in the preparation are large (up to .5 mm in diameter) making biochemical studies on single, identifiable neurons possible.

(Up to 30 neurons in the ganglion are identifiable in the sense that they can be recognized from preparation to preparation by their size, morphology, and position, and have been shown to have characteristic patterns of electrical activity (Frazier, et al., 1967). Many have been shown to have characteristic responses to acetyl choline (Frazier, et al., 1967),

characteristic amounts of choline acetyltransferase (McCaman & Dewhurst, 1970; Giller & Schwartz, 1971a), and acetylcholinesterase (Giller & Schwartz, 1971b) activities, and characteristic patterns of incorporation of amino acids into their proteins as studied on SDS polyacrylmide gels (Wilson, 1971).

(3) The electrical activity of the identifiable neurons is easily monitored. The effect of various manipulations on the electrical activity of particular neurons can be observed.

(4) Particular neurons can be manipulated electrophysiologically in specific ways, for example, by stimulation of known presynaptic pathways or by intracellular current injections.

SUMMARY

Changes in protein synthesis may play an important role in the function of the nervous system. In order to study one aspect of this, I have been doing research on the effect of high $[K^+]$ media on protein synthesis in the parieto-visceral ganglion of Aplysia californica.

Previous studies have shown that the metabolism of proteins in brain is changed in animals exposed to novel situations, including sensory stimulation, forced motor activity, electroconvulsive shock, induced convulsions, and learning situations. The studies discussed in this introduction are summarized in Table I.

TABLE I. IN VIVO STUDIES OF PROTEIN SYNTHESIS IN BRAIN

Type of situation	Animal	Effect[1]	Reference
Sensory stimuli			
exposure to light	rabbit	inc. incorp.	Talwar, et al. (1966)
exposure to light	Rhesus monkey	inc. incorp.	Singh & Talwar (1967)
exposure to light	Rhesus monkey	inc. incorp.,S-100	Singh & Talwar (1969)
unilateral eye removal	frog	inc. incorp. tectum contralateral to remaining eye	Wegener (1970)
unilateral eye removal	chick	inc. incorp. contralateral to remaining eye	Bondy & Margolis (1970)
first exposure to light at age day 50	rat	inc. incorp. initially; dec. incorp. subsequently	Rose (1967)
exposure to light	chick	inc. incorp. roof of forebrain	Bateson, et al. (1969)

TABLE I. (CONTINUED)

Type of situation	Animal	Effect[1]	Reference
Motor activity			
forced running	rat	inc. incorp. brain and spinal motoneurons	Altman (1963)
forced running	rat	inc. incorp. motor cortex	Rose (1967)
forced swimming	rat	inc. incorp.	Krawczynski (1961)
forced swimming	rat	dec. incorp. spinal motoneurons	Jakoubek & Gutmann (1968)
forced running	rat	dec. incorp. cerebral cortex	Tiplady (1972)
Convulsions			
electroconvulsive shock	mouse	dec. incorp.	Dunn & Guiditta (1971), Dunn, et al. (1971)
Spreading depression	rat	dec. incorp.	Ruscak (1964), Bennet & Edelman (1969)

TABLE I. (CONTINUED)

Type of situation	Animal	Effect[1]	Reference
Water deprivation	guinea pig	inc. incorp. into vasopressin	Takabatake & Sachs (1964)
Hemorrhage	guinea pig	no effect on vasopressin	Sachs, et al. (1967)
Learning situations			
non-preferred paw	rat	inc. S-100, CA3 region	Hyden & Lange (1970a,b)
non-preferred paw	rat	inc. incorp. spec., hippocampal pyramidal cells	Hyden & Lange (1968)
non-preferred paw	rat	inc. incorp. CA3 region	Yanagihara & Hyden (1971)
non-preferred paw	rat	dec. incorp. hippocampus	Hyden & Lange (1972)
leg-lift	cockroach	inc. incorp. spec.	Kerkut, et al. (1972)
tentacle retraction	snail	inc. incorp. spec.	Emson, et al. (1971)

TABLE I. (CONTINUED)

Type of situation	Animal	Effect[1]	Reference
Learning situations			
dark avoidance	rat	inc. spec.	Ungar, et al. (1972)
airpuff habituation, loud sound habituation, & stepdown avoidance	rat	inc. spec.	Ungar (1971)
Other reviews			Glassman (1969) Uphouse,et al. (1973)

[1]Abbreviations have the following meanings: inc. incorp., increased incorporation of a radioactive precursor into total protein; dec. incorp., decreased incorporation; inc. incorp. spec., increased incorporation into a specific fraction or fractions of protein; inc. spec., increase of the total amount of some specific protein.

In vitro studies of brain tissue (mostly brain slices of vertebrate brain) showed changes in protein synthesis in tissue which was electrically stimulated or exposed to high $[K^+]$ media. Both treatments generally caused a decrease in protein synthesis, which was similar to the change in brain protein synthesis occurring during convulsive activity. These studies are summarized in Table II. An unanswered question in these *in vitro* experiments was whether all proteins in the tissue were affected equally by stimulation. To study this question, I chose to stimulate nervous tissue using high $[K^+]$ media.

The rationale for using the high $[K^+]$ technique was that (1) the resting membrane potential of both neurons and glial cells depends largely on external $[K^+]$; (2)electrical activity causes an efflux of intracellular K^+, raising extracellular $[K^+]$ and thereby mimicking some effects of using high $[K^+]$ media; (3) electrical stimulation and raising external $[K^+]$ have similar effects in brain tissue not only on protein synthesis but also on respiration and glycolysis, high energy phosphate metabolism, cyclic adenosine monophosphate metabolism, and neurotransmitter and neurohormone release; and (4) functional roles for the K^+ released during electrical activity have been suggested.

The parieto-visceral ganglion of *Aplysia californica* was chosen for these studies because (1) long term *in vitro* experiments can be done with relative ease,

TABLE II. IN VITRO EXPERIMENTS ON PROTEIN SYNTHESIS IN NEURAL TISSUES

Type of stimulus	Tissue used	Effect	Reference
Electrical stimulation	brain slice	dec. incorp.	Orrego & Lipmann (1967)
Acidic amino acids	brain slice	dec. incorp.	Orrego & Lipmann (1967)
Electrical stimulation	brain slice	dec. incorp.	Jones & Banks (1970)
High $[K^+]$	brain slice	dec. incorp.	Jones & McIlwain (1971)
High $[K^+]$	brain slice	dec. incorp.	Folbergrova (1961)
High $[K^+]$	brain slice	dec. incorp.	Mase, et al.(1962)
High $[K^+]$	brain slice	inc. incorp. at moderate levels, dec. incorps at high levels	Blomstrand & Hamberger (1970)
Electrical stimulation	superior cervical ganglion	dec. incorp. (relative to incorp. in amino acid pool)	McBride & Klingman (1972)
Synaptic stimulation	giant cell, Aplysia	no change	Wilson & Berry (1972)

(2) studies on identifiable neurons or groups of neurons can be done, and (3) electrical activity of identifiable neurons can be monitored or manipulated electrophysiologically.

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CHAPTER II

HIGH K^+ EFFECTS ON LEUCINE INCORPORATION IN *APLYSIA* NERVOUS TISSUE. GEL ELECTROPHORESIS STUDIES

Much work has been done to investigate the possible coupling of electrical activity and behavior to macromolecular metabolism in the nervous system (for review, see Glassman, 1969, and introduction to this thesis). Recent work describing electrophysiological and biochemical aspects of the parieto-visceral ganglion (PVG, or abdominal ganglion) (Frazier, 1967; Strumwasser, 1971) has laid the groundwork for investigating further the effects of electrical activity in neurons on their synthesis of macromolecules. Synaptic stimulation of R2, the giant cell of the PVG, caused an increase of labeled uridine incorporation into RNA (Berry, 1969; Peterson & Kernell, 1970; Kernell & Peterson, 1970; Berry & Cohen, 1972); however, the interpretation of this result as an increase in RNA synthesis is problematical since the increased incorporation is not found at lower uridine concentrations (Wilson & Berry, 1972). Using similar stimulation procedures, Wilson & Berry (1972) also found no change in the spectrum of proteins synthesized by R2.

In the present work, a more general and more prolonged stimulation of the cells of the PVG was sought by

the use of high $[K^+]$ media. Since the membrane potential of nerve cells and glia is largely determined by the distribution of K^+ across their membranes, an effective depolarization of these cells is produced by raising external K^+ levels (Carpenter & Alving, 1968). Thus, high $[K^+]$ media can be used as a gross means of depolarizing cells for studies aimed at finding biochemical processes coupled to the membrane polarization. In vertebrate brain slices, high $[K^+]$ usually decreases amino acid incorporation into protein (Folbergrova, 1961).

Whole PVGs were labeled in a defined medium containing a labeled amino acid and either normal or higher than normal levels of K^+. The proteins synthesized were analyzed on polyacrylamide gels for differences due to changes in K^+ concentration in the incubation medium. To maximize the chance of seeing any changes produced, a double-label technique was used. This chapter shows that in high $[K^+]$ media significant changes in the relative amount of incorporation occurred, including a previously reported change at 50,000 molecular weight (Ram, 1972).

MATERIALS AND METHODS

1. Animal

Adult Aplysia californica, obtained from Pacific Bio-Marine, Venice, California, were maintained at 13-14°C in large tanks containing filtered and aerated sea water.

The animals were kept on a 12 hr light : 12 hr dark light cycle, and experiments were generally begun within three hours of the time the lights went on. Following a pedal incision, the nerves of the PVG were cut and the ganglion was lifted out by the branchial nerve (Eales, 1921). In all subsequent transfers the ganglion was held by the branchial nerve; in the most recent experiments, a thread was tied to the branchial nerve and used for handling the ganglion. The left and right connectives were generally cut about half-way between the PVG and the anterior ganglia. All dissections and incubations were carried out in a room thermostatically held at 14°C.

2. Incubation protocols and sample preparation

The usual incubation protocol, called parallel control, is shown schematically in Figure 1: Two ganglia, designated here the control PVG and the experimental PVG, were dissected from two animals of approximately the same weight and sexual maturity [determined from the weight of the reproductive tract (Strumwasser, et al., 1969)]. The control ganglion went through two 90 minute rinses in normal blood salts medium (Table I) followed by a 240 minute incubation in normal incubation medium (Table I), which usually contained ^{14}C-leucine. The experimental ganglion went through two 90 minute rinses in experimental blood salts medium (Table I) followed by a 240 minute

Figure 1. Schematic diagram of parallel control incubation protocol and sample preparation.

SAMPLE PREPARATION

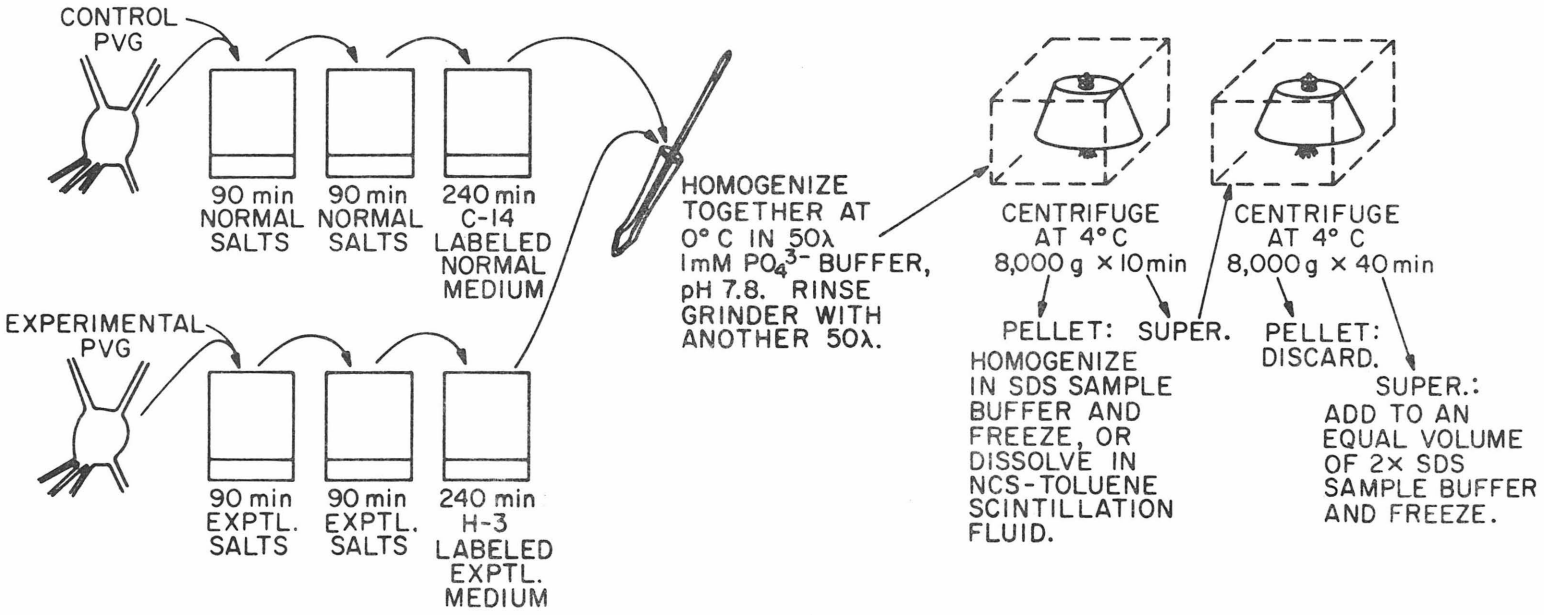

TABLE I.

COMPOSITIONS OF MEDIA

Medium	Constituents, mM[1]									Other constituents[4]
	Na	K	Ca	Mg	SO_4	Cl	Tris	Glucose	Leucine[3]	
Normal blood salts	476	10	13	49	28	554	0	0	0	-
Experimental blood salts	476-X	10+X	13	49	28	554	0	0	0	-
Normal incubation medium	476	10	13	49	28	554[2]	10	55	1.06×10^{-2}	+
Experimental incubation medium	476-X	10+X	13	49	28	554[2]	10	55	1.06×10^{-2}	+

[1]All media prior to addition of radioactive leucine were prepared either from sterile stock solutions or were filtered through a .22 u Millipore filter. Media were pipetted into clean glass vials for use. No bacterial contamination was found when aliquots were plated onto petri dishes containing a rich bacterial growth medium (glucose, 10 g/l; bactopeptone, 10 g/l; yeast extract, 1 g/l; $MgSO_4$, 1 g/l; KH_2PO_4, 1.5 g/l; K_2HPO_4, 1 g/l; and agar, 20 g/l; adjusted to pH 6.4-6.5). Incubations were carried out in .75 ml of incubation media.

[2]Plus enough Cl added as HCl to Tris stock solution to give pH 7.8.

[3]Calculated leucine concentration of 2.5 uCi of ^{14}C-leucine/.75 ml incubation medium (L-leucine ^{14}C , 312 mCi/mmole, Schwarz/Mann Div., Becton, Dickenson & Co., Orangeburg, N.Y.). Cold leucine

TABLE I. (CONTINUED)

was added to ^{3}H-leucine (approximately 50 uCi/.75 ml incubation medium) to yield the same leucine concentration (L-leucine-4,5-^{3}H, 56 Ci/mmole, same manufacturer). (However, see Appendix B).

[4]Vitamins, amino acids, $PO_4^{\equiv}$ (as Na salt, pH 7.8, final concentration .13 mM), and HCO_3^- (Na salt, 1.6 mM). Vitamins and amino acids are those used for Eagle's (1969) minimum essential medium (MEM): L-glutamine at 1/15 MEM concentration, essential amino acids (except leucine) at 1/75 MEM concentration, non-essential amino acids at 2/15 MEM concentration, and vitamins at 2/3 MEM concentration.

incubation in experimental incubation medium (Table I), which usually contained ^{3}H-leucine.

After a brief rinse, nerves were removed, and the two ganglia were homogenized together on ice in 50 µl .001 M phosphate buffer (pH 7.8) for 1 minute in a hand held glass-glass homogenizer. The homogenizer was rinsed with another 50 µl of the phosphate buffer, and the combined sample was then centrifuged at 8000 g for 10 minutes (<4°C). The supernatant was centrifuged again at 8000 g for 40 minutes (<4°C), and the final supernatant, called here the aqueous soluble fraction, was combined with an equal volume of 2X sodium dodecyl sulfate (SDS) sample buffer (double the concentration of normal SDS sample buffer, which contains 10% glycerol, .2% SDS, 2% 2-mercaptoethanol, .0015% bromphenol blue, and .01 M Na^{+}-phosphate buffer, pH 7.2 (Arch, 1972)) and frozen at -10°C for later analysis. Sometimes the pellet from the first centrifugation was further homogenized in normal SDS sample buffer and frozen for later analysis.

A second incubation protocol, called <u>internal control</u>, utilized only one ganglion. The ganglion was ^{14}C labeled in the usual manner for a control ganglion. This was followed by three 20 minute rinses in normal blood salts and then the ganglion was put through the usual procedure for ^{3}H labeling an experimental ganglion. Homogenization and centrifugation was as for <u>parallel control</u>

experiments except the volumes used were cut in half. Analysis of the three post-^{14}C incubation rinses showed that about 75% of the counts that came out in the 60 minute rinse came out in the first 20 minutes.

In a third protocol, called simultaneous incorporation, a ganglion was treated either as the control or the experimental ganglion in Figure 1; however, both ^{14}C- and ^{3}H-leucine were present during the incubation period. This protocol was used to demonstrate the similarity in ^{3}H- and ^{14}C-incorporation patterns.

3. SDS-polyacrylamide gel electrophoresis

After thawing, samples were heated to 65°C for 30 minutes and centrifuged at 8000 g for 10 minutes (<4°C). The electrophoresis system was a standard one for SDS-polyacrylamide gels, modeled after the system of Shapiro (1967), as described by Burgess (1968). Peptides are separated on SDS-polyacrylamide gel approximately according to molecular weight. Tubes were 3 mm i.d. and generally pretreated with Siliclad (Becton, Dickinson & Co., Parsippany, N. J.); gels were 75 mm long; and electrophoresis took about 5 hours at a constant voltage of 28 V. No more than 20 λ of the sample was used per gel. After electrophoresis, the position of the tracking dye (bromphenol blue) relative to the top and bottom of each gel was noted. Gels were stained for several hours in 0.25% Coomassie Brilliant Blue in 9% acetic acid, 45% methanol. The gels were destained by

diffusion in 7.5% acetic acid, 5% methanol for several days and then traced on a Joyce, Loebl and Co. (Gateshead, England) microdensitometer. Standards used for determination of the molecular weight scale of the gels were bovine serum albumin (68K, K=1000 daltons), human gamma globulin (H-chain, 52K; L-chain, 24K), ovalbumin (45K), bovine chymotrypsinogen (26K), and horse heart cytochrome c (11.7 K). (See Appendix A). Molecular weights of peaks in experimental patterns are given to the nearest 5K. The actual molecular weights are expected to agree with these within about 10% (Weber & Osborn, 1969).

4. Radioactive gel counting

Gels were sliced with an "egg-slicer", consisting of a frame with parallel 40-gauge (.003 in) wires evenly spaced and a slotted trough in which the gel was placed. Slices were approximately 1-1/4 mm thick, and the standard deviation of slice thickness (measured by slicing a uniformly labeled gel[1]) was about 8%. The position of stained bands was noted before taking the slices from the slicer. Slices were placed in 5 ml of a toluene based counting cocktail containing Nuclear Chicago Solubilizer (NCS)

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[1]To make this gel, 50 λ of the aqueous soluble fraction of a ^{3}H labeled ganglion (total volume of fraction equaled 90 λ) was added to a polyacrylamide gel solution before gelling. The solution was mixed well, and gels were formed as usual. One of these gels was extruded, sliced, and counted (2 σ counting error in each slice was less than 2%).

(Ward, et al. 1970), and counted on a Beckman LS200 liquid scintillation counter.

Calculations and plotting were done on an IBM batch-processor. After correcting for background and overlap between channels, bar graphs of the control counts, of the experimental counts, and of the ratio of experimental to control counts were plotted. A peak appeared in the ratio bar graph where a relative increase in experimental counts, compared to control counts, occurred. The size of a peak on the ratio bar graph was determined using the following equation:

$$\text{Peak size on ratio bar graph} = \frac{R_{1peak} + R_{2peak}}{R_{left} + R_{right}}$$

where R_{ipeak} are the ratios of counts in two slices at the peak, R_{left} is the ratio of counts in the second slice to the left of the peak, and R_{right} is the ratio of counts in the second slice to the right of the peak.

Most of the data given in this paper are for individual gels which showed typical results. The number (N) of replicates of each type of experiment is indicated in the text. For particularly important points, statistical analyses of all the relevant data are shown. Results are given as the mean $\pm$ standard deviation, except when otherwise stated.

5. Determination of specific activity of proteins

To analyze aqueous soluble fractions, one ml of ice-cold 10% trichloracetic acid (TCA) was added to the sample to be analyzed. After standing on ice for at least five minutes, the sample was centrifuged at 8000 g for 10 minutes ($<4^{o}C$). To count the TCA soluble radioactivity a 100 λ aliquot of the supernatant was put in a scintillation vial, evaporated to dryness at $60^{o}C$, and 10 ml of counting cocktail containing NCS (Ward et al., 1970) was added. The rest of the supernatant was pipetted off, and the pellet was washed twice more with ice cold 10% TCA. Protein in the pellets was determined by the method of Lowry, et al. (1951). To determine the radioactivity in the measured protein, 100 λ of the final Lowry solution was pipetted into a scintillation vial, evaporated to dryness, and counted in 10 ml of counting fluid containing NCS (Ward, et al., 1970). Amino acyl tRNA would be included in this solution, but due to the long incubation periods used, this is not a serious contaminant. According to Schwartz, et al. (1971), after a one hour incorporation, the amount of labeled amino acid in the tRNA fraction was less than 5% of the amount in protein. Control tests showed that the color of the Lowry solution did not affect the counting efficiency.

The procedure for analyzing aqueous insoluble pellets was the same except that the first centrifugation

was done with one ml of phosphate buffer (pH 7.8) added to the sample instead of 10% TCA.

RESULTS

1. Normal pattern of staining and incorporation

Figure 2 shows the staining pattern of the aqueous soluble fraction of the PVG and the normal incorporation pattern obtained with ^{14}C-leucine and ^{3}H-leucine. Particularly noticeable landmarks in the staining pattern are the three bands, identified as bands, a, b, and c in Figure 2a, which occur at approximately 50K (K = 1000 daltons), 40K, and 35K, respectively. The 50K peak ran between the H chain of gamma globulin (52K) and ovalbumin (45K), when these markers were added to a ganglion sample. (See Appendix A). The ^{14}C- and ^{3}H- patterns shown in Figures 2b and 2c are from a simultaneous incorporation experiment in normal media (N = 3). As expected, they are quite similar. The incorporation pattern shows two prominent peaks, located at approximately 50K and 40K. The 35K staining peak generally fell in a trough in the incorporation pattern.

2. Effect of high [K^+] media on PVG

High [K^+] media ([K^+] = 90-110 mM) caused changes in the relative amounts of incorporation into various proteins observed on these gels. The most prominent change occurred in the 50K peak. A typical result for a parallel control experiment in which experimental media had 90 mM

Figure 2. Results of a simultaneous incorporation experiment in normal media. (a) Staining pattern of aqueous soluble fraction of PVG. Bands a, b, and c are approximately 50K, 40K, and 35K respectively. (b) ^{3}H incorporation pattern. (c) ^{14}C incorporation pattern. (d) Ratio of ^{3}H counts to ^{14}C counts in each slice. Error bars shown for several slices are the 95% confidence limits. Total counts in gel: ^{3}H, 23600 cpm, ^{14}C, 4013 cpm.

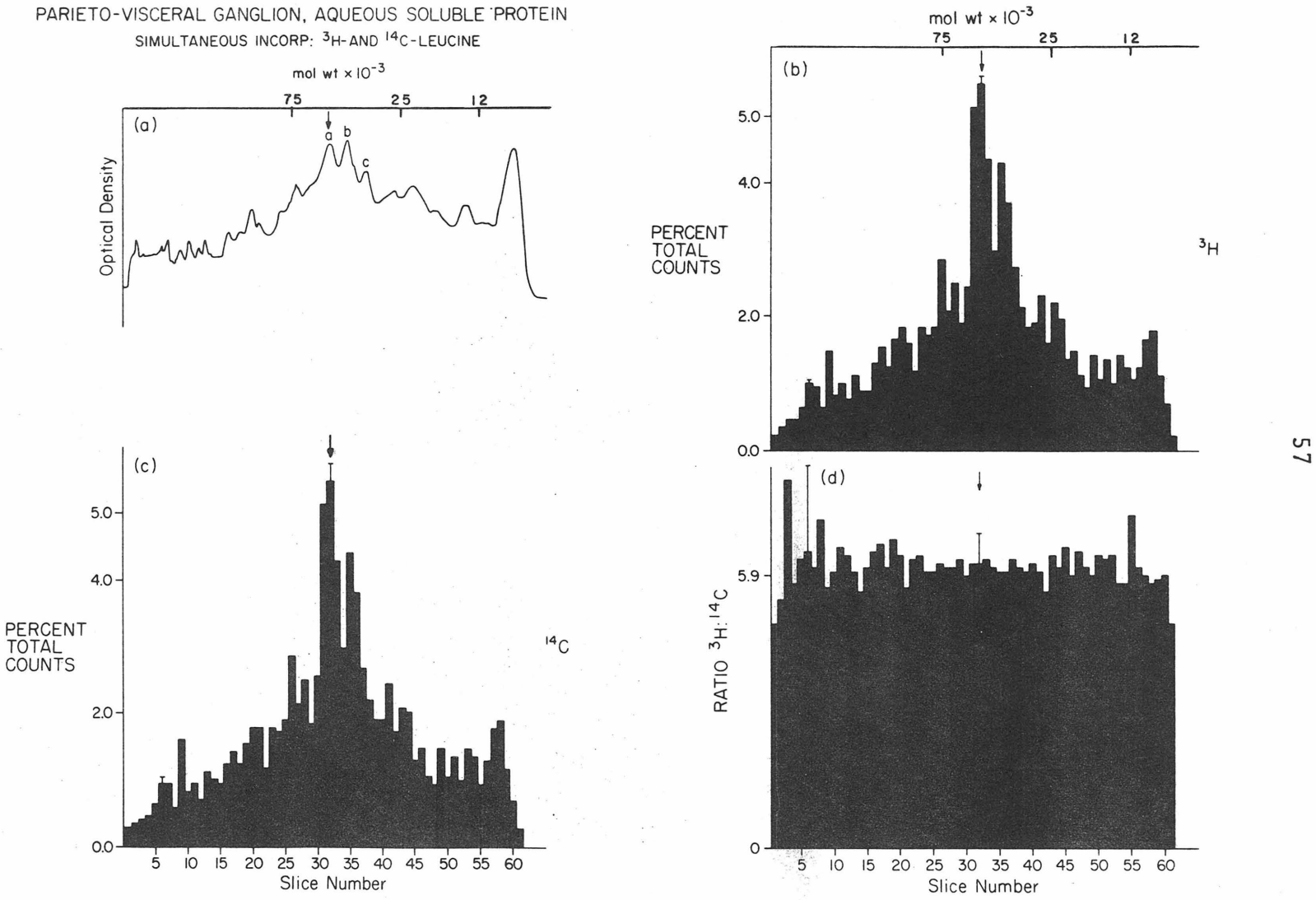
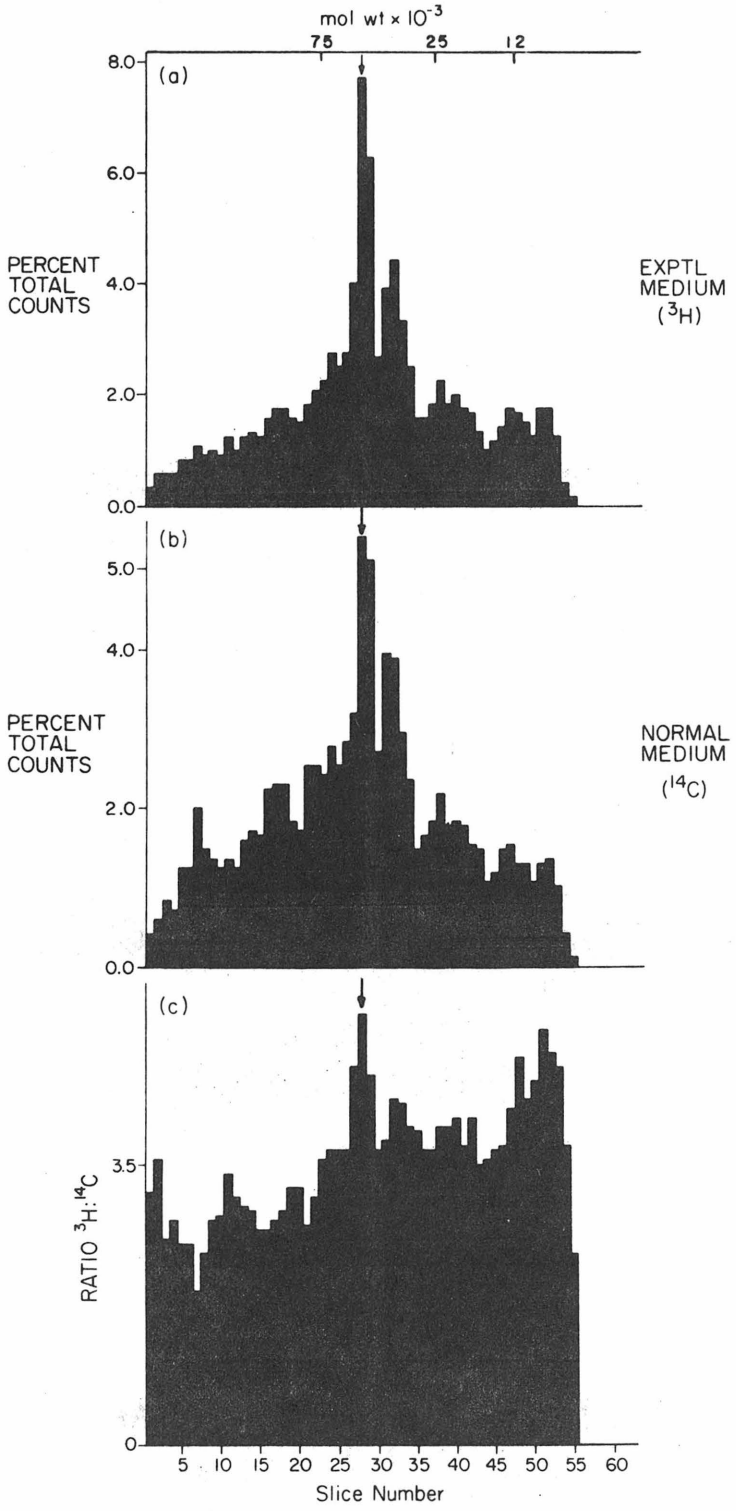

PARIETO-VISCERAL GANGLION, AQUEOUS SOLUBLE PROTEIN
SIMULTANEOUS INCORP: ^{3}H- AND ^{14}C-LEUCINE
mol wt x 10^{-3}
75
25
12
(a)
a
b
c
Optical Density
(b)
PERCENT TOTAL COUNTS
^{3}H
5.0
4.0
2.0
0.0
(c)
PERCENT TOTAL COUNTS
^{14}C
5.0
4.0
2.0
0.0
(d)
RATIO ^{3}H:^{14}C
5.9
0
5 10 15 20 25 30 35 40 45 50 55 60
Slice Number

Figure 3. Patterns of aqueous soluble fraction of PVG obtained in a parallel control experiment in which the ^{3}H pattern was from a PVG incubated in experimental media (90 mM $[K^{+}]$), and the ^{14}C pattern was from a PVG incubated in normal media. (a) ^{3}H incorporation pattern. (b) ^{14}C incorporation pattern. (c) Ratio of ^{3}H counts to ^{14}C counts in each slice. The staining pattern in these experiments was essentially the same as the pattern shown in Figure 2a. Total counts in gel: ^{3}H, 28885 cpm; ^{14}C, 7856 cpm.


PARIETO-VISCERAL GANGLION, AQUEOUS SOLUBLE PROTEIN
EXPTL MEDIUM: 90 mM K+
mol wt x 10-3
75
25
12
(a)
8.0
6.0
4.0
2.0
0.0
PERCENT TOTAL COUNTS
EXPTL MEDIUM (3H)
(b)
5.0
4.0
2.0
0.0
PERCENT TOTAL COUNTS
NORMAL MEDIUM (14C)
(c)
3.5
0
RATIO 3H:14C
5 10 15 20 25 30 35 40 45 50 55 60
Slice Number

$[K^+]$ is shown in Figure 3 (N = 6). In this case, the percent of total counts in the gel appearing in the three slices centered at 50K increased from 13.7% in the control ganglion to 18.2% in the experimental ganglion.

Figure 3c shows a plot of the ratio of experimental to control counts in each slice of the gel. The ratio of experimental to control counts at the center of the 50K peak was nearly 1.5 times the ratio of experimental to control counts two slices to either side. Differences of smaller magnitude occurred at 40K, 25K, and 5-15K.

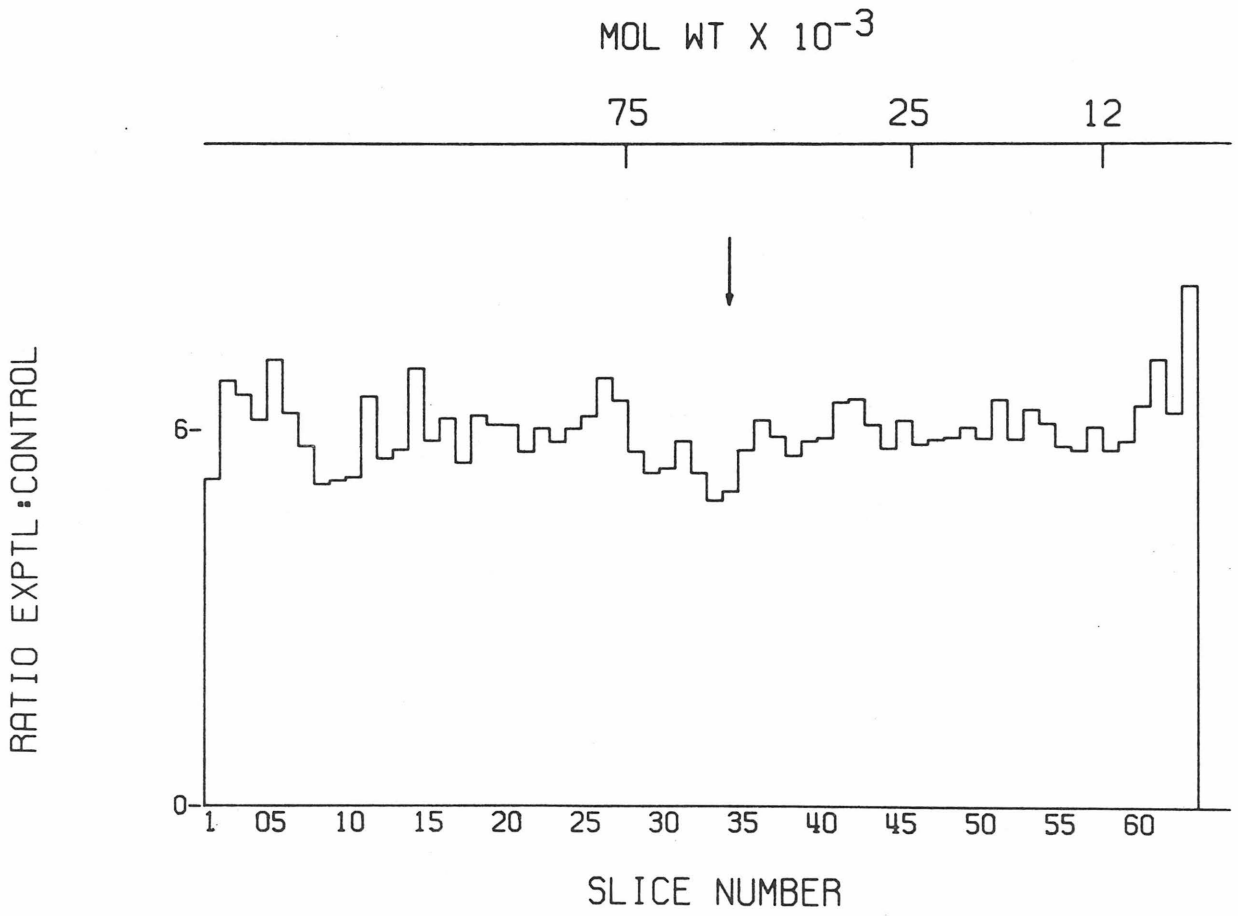
In order to analyze these peaks statistically, ratio patterns obtained from parallel control experiments in which the experimental media had high $[K^+]$ (90-110 mM $[K^+]$, N = 10) were compared to ratio patterns obtained from parallel control experiments in which the experimental media had normal levels of K^+ (Figure 4, N = 9). "Peaks" were located with reference to the incorporation pattern of the control ganglion. Operationally, the two slices in the 50K peak were the slice with the maximum counts located at 50K and the slice to its left.

Similarly, the two slices in the 40K peak were the slice with the maximum number of counts located at 40K and the slice to its right, except when the slice to the right of the 40K relative maximum was greater than 70% of the maximum, as is the case in Figure 3. In such cases the two slices to the right of the relative maximum at 40K were

Figure 4. Ratio of ^{3}H (experimental) counts to ^{14}C (control) counts for aqueous soluble fraction of PVG from a parallel control experiment in which the experimental media and the control media both contained 10 mM $[K^{+}]$.
Total counts in gel: ^{3}H, 28210 cpm; ^{14}C, 5187 cpm.

PARIETO-VISCERAL GANGLION, AQUEOUS SOLUBLE PROTEIN

EXPTL MEDIUM: NORMAL K^+



taken as the peak. These algorithms placed the "50K peak" at or slightly to the left of the 50K staining peak, and the "40K peak" in the trough between the 40K and 35K staining peaks. An algorithm to locate the 25K and 5-15K peaks reliably based on the control incorporation pattern or the staining pattern could not be obtained, even by broadening the definition of a peak to include three slices.

The size of the 50K and 40K peaks on the ratio bar graph were calculated for both high $[K^+]$ and normal $[K^+]$ experiments using the equation given in Materials and Methods. Results for the 50K and 40K peaks are shown in Table II. For both molecular weights, peak size on the ratio bar graph was significantly greater in high $[K^+]$ experiments than for normal $[K^+]$ experiments ($p < .01$ for both, Student's t test and Mann-Whitney U test). The strongest effect, at 50K, received the most attention in subsequent analysis.

The 50K peak was analyzed by two additional calculations: (a) the average ratio of the two slices at the peak (located as described above) was divided by the ratio of the total experimental counts in the gel to the total control counts in the gel. The results, shown in Table III, demonstrated that the change in the "peak size" was actually due to a relative increase in incorporation at 50K compared to most proteins, and not due to a relative decrease in the incorporation just at the slices adjacent

TABLE II.

SIZE OF 50K AND 40K PEAKS ON RATIO BAR GRAPH[1]

Peak	50K		40K	
Type of sample[2]	PVG-sol Normal	PVG-sol High [K^+]	PVG-sol Normal	PVG-sol High [K^+]
Size of peak[3]	1.05	1.50	1.01	1.12
$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
standard deviation	.14	.15	.07	.07
(N)	(9)	(10)	(9)	(10)

[1]The size of the peak was determined as described in Materials and Methods. Peaks were located as described in the text. For ratios of two identical samples, the peak size should be 1.0.

[2]PVG-sol is the aqueous soluble fraction of the parieto-visceral ganglion. These results are from parallel control experiments. "Normal" means the experimental ganglion was in normal [K^+]; "High [K^+]" means the experimental ganglion was in media with 90-110 mM [K^+].

[3]For both 50K and 40K peaks the size of the peak in high [K^+] experiments was significantly greater than the size of the peak in normal [K^+] experiments ($p<.01$, Student's t test and Mann-Whitney U test).

TABLE III.

50K PEAK: $\frac{\frac{1}{2}(R_{1peak} + R_{2peak})^*}{R_{total}}$

Type of sample	PVG-sol Normal	PVG-sol High $[K^+]$
$\frac{\frac{1}{2}(R_{1peak} + R_{2peak})}{R_{total}}$	1.03	1.46**
$\pm$	$\pm$	$\pm$
standard deviation	.10	.17
(N)	(9)	(10)

*R_{ipeak} are the ratios of experimental to control counts at two slices located at 50K (located as described in the text). R_{total} is the ratio of total experimental counts in the gel to total control counts in the gel.

**significantly different ($p<.01$, Student's t test).

to 50K. (b) The percent of total counts in the gel appearing in the two slices located at 50K (again located as described above) was determined for both the experimental pattern and the control pattern. The percent of total counts appearing in these two slices increased from $9 \pm 2\%$ for label incorporated in normal media to $13\frac{1}{2} \pm 2\%$ for label incorporated in high $[K^+]$ media. This difference was highly significant ($p < .01$, Student's t test). These results are shown in Table IV.

The relative increase of incorporation at 50K was initially discovered using high $[K^+]$ solutions containing 106 mM $[K^+]$ and was subsequently confirmed at 110 mM $[K^+]$ and 90 mM $[K^+]$. When $[K^+]$ was lowered to 50 mM, the enhancement at 50K disappeared as did most of the other effects of high $[K^+]$ (Figure 5, N = 3).

To check whether high $[K^+]$ caused a general increase in the relative incorporation into 50K peptides in the ganglia, the SDS solubilized fraction of the aqueous insoluble pellet from the 8000 g x 10' centrifugation was analyzed by gel electrophoresis. No prominent peak at 50K occurred in the ratio pattern, thereby showing that the effect did not occur with all 50K peptides (Figure 6, N = 5).

Two experiments dealt with the question of whether this was an isotope specific effect. In one, ^{3}H-leucine was used in the control medium and ^{14}C-leucine was used in

TABLE IV.

PER CENT TOTAL COUNTS AT 50K[1]

Type of experiment[2]	Normal		High $[K^+]$	
Type of sample	PVG-sol Normal ^{14}C	PVG-sol Normal ^{3}H	PVG-sol Normal ^{14}C	PVG-sol High $[K^+]$ ^{3}H
% total counts[3] at 50K	8.9	9.2	9.3	13.6
$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
standard deviation	2.3	2.2	1.8	1.8
(N)	(9)	(9)	(10)	(10)

[1]50K was located as described in text. The number of counts appearing in the two slices at 50K were divided by the total number of counts appearing in the gel and then multiplied by 100%.

[2]Experiments have been separated into two groups in which the experimental incubation was in Normal medium or High $[K^+]$ medium.

[3]PVG-sol, High $[K^+]$, ^{3}H is significantly different from all others ($p<.01$), all of which are not significantly different from each other (Student's t test, unpaired comparison). A paired comparison of the data for Normal $[K^+]$ experiments showed that PVG-sol, Normal, ^{3}H is not significantly higher than PVG-sol, Normal, ^{14}C ($p>.2$, Student's t test for paired variates).

Figure 5. Ratio of ^{3}H (experimental) counts to ^{14}C (control) counts for aqueous soluble fraction of PVG from a parallel control experiment in which the experimental media contained 50 mM $[K^{+}]$. Total counts in gel: ^{3}H, 23728 cpm; ^{14}C, 5544 cpm.

PARIETO-VISCERAL GANGLION, AQUEOUS SOLUBLE PROTEIN

EXPTL MEDIUM: 50 MM K^+

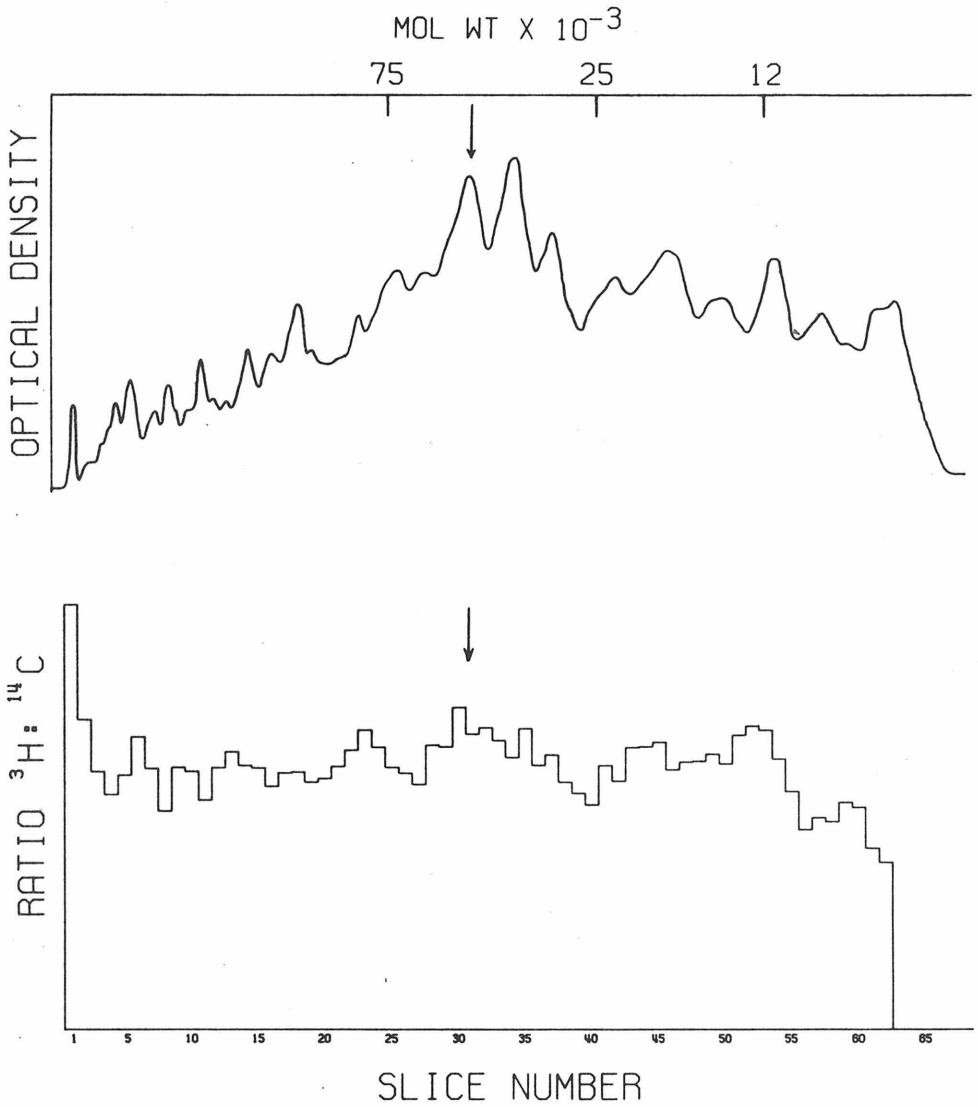
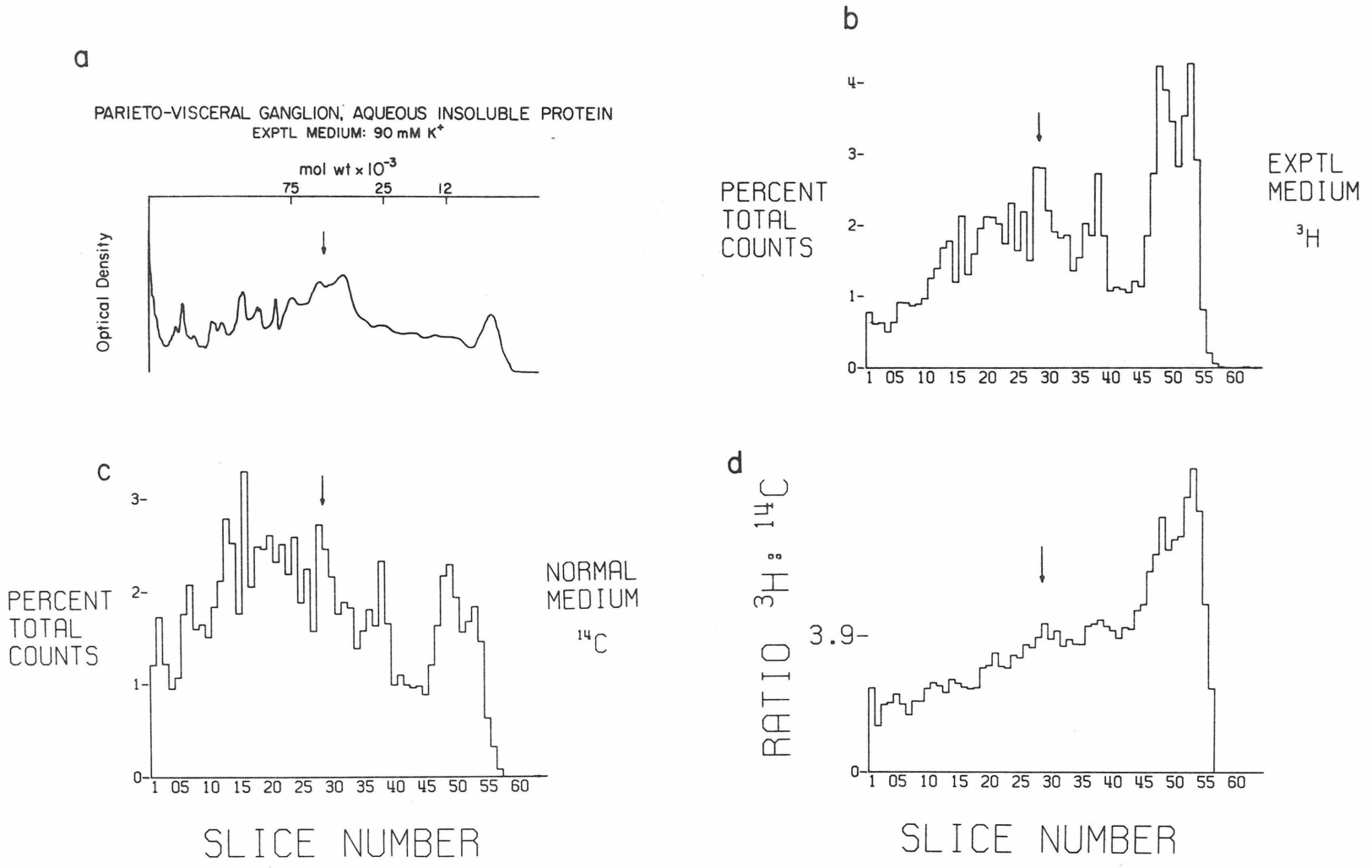


Figure 6. Patterns of aqueous insoluble fraction of PVG obtained in a parallel control experiment in which the ^{3}H pattern was from a PVG incubated in experimental media (90 mM [K^{+}]), and the ^{14}C pattern was from a PVG incubated in normal media. (a) Staining pattern. (b) ^{3}H incorporation pattern. (c) ^{14}C incorporation pattern. (d) Ratio of ^{3}H counts to ^{14}C counts in each slice. Total counts in gel: ^{3}H, 16220 cpm; ^{14}C, 4178 cpm.


a
PARIETO-VISCERAL GANGLION, AQUEOUS INSOLUBLE PROTEIN
EXPTL MEDIUM: 90 mM K+
mol wt x 10-3
75
25
12
Optical Density
b
PERCENT TOTAL COUNTS
EXPTL MEDIUM
3H
c
PERCENT TOTAL COUNTS
NORMAL MEDIUM
14C
d
RATIO 3H : 14C
3.9
SLICE NUMBER
SLICE NUMBER

the experimental medium. The 50K peak size on the ratio bar graph for this experiment was 2.3, which is actually a bit larger than is usually observed with the usual parallel control protocol.[1] The other experiment was a simultaneous incorporation experiment in which high $[K^+]$ (90 mM $[K^+]$) was used throughout. The ^{14}C- and ^{3}H-patterns were affected identically. The 50K peak size on the ratio bar graph in this experiment was 1.01 (i.e. no peak present), and the percent total counts at 50K in both patterns was in the range expected for high $[K^+]$ patterns (12.0% for ^{3}H and 12.2% for ^{14}C).

3. Effect of lowering $[Na^+]$ without raising $[K^+]$

Since equimolar reductions of $[Na^+]$ were made whenever $[K^+]$ was raised, the "high $[K^+]$" effect could conceivably be a "low $[Na^+]$" effect. To test this, $[Na^+]$ was reduced by 80 mM (the amount it was reduced when raising $[K^+]$ from 10 mM to 90 mM) and replaced with equimolar amounts of $tris^+$, neutralized to pH 7.7 with HCl. A typical ratio bar graph for a "low $[Na^+]$, normal $[K^+]$" experiment is shown in Figure 7 (N = 3). No relative increase in incorporation at 50K is apparent. In fact, this "low $[Na^+]$" treatment produced a small, but significant decrease in the

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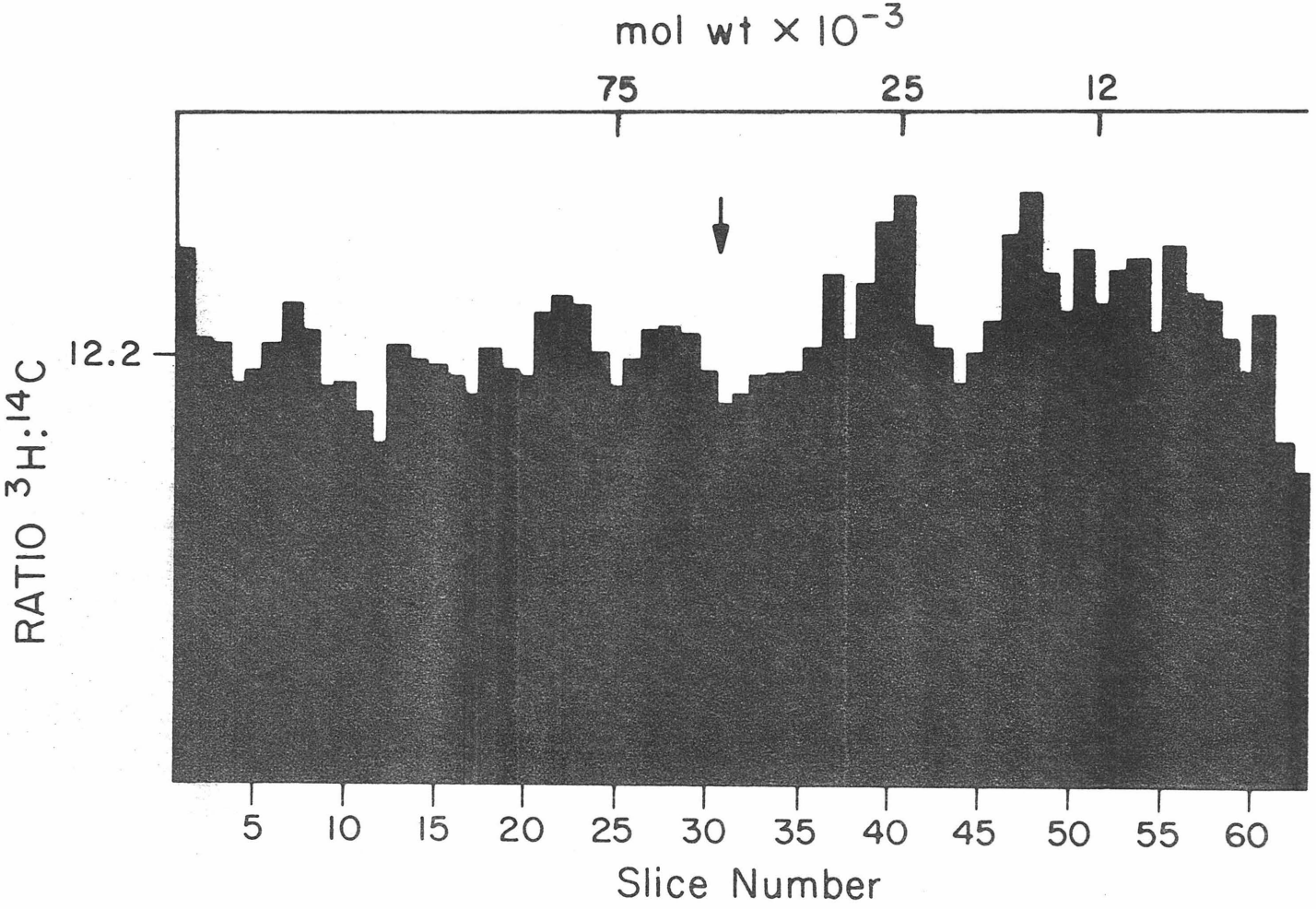
[1]The standard deviation of this peak size due to counting error was large in this experiment, due to an unusually small number of counts obtained in the ^{14}C labeled ganglion. The peak size was 2.3 ± 0.6.

Figure 7. Ratio of ^{3}H (experimental) counts to ^{14}C (control) counts for aqueous soluble fraction of PVG from a parallel control experiment in which $[Na^{+}]$ in the experimental media had been reduced by 80 mM and replaced with equimolar amounts of $tris^{+}$ (neutralized to pH 7.7 with HCl). Total counts in gel: ^{3}H, 56055 cpm; ^{14}C, 4604 cpm.

PARIETO-VISCERAL GANGLION, AQUEOUS SOLUBLE PROTEIN
EXPTL MEDIUM: Reduced Na^+ (80 mM reduction; tris substitution)



relative amount of incorporation at 50K.

4. Internal control protocol: a test for a post-synthesis effect

The internal control protocol was originally designed to test the possibility that the high [K^+] effect was due to some aberrant effect on the normal peptides in the ganglion after they were synthesized, rather than due to a change in the control of their synthesis. For example, high [K^+] might be causing the breakdown of high molecular weight peptides into 50K and other lower molecular weight peptides. In the internal control experiment, ganglia were first labeled with ^{14}C-leucine in normal medium and then subsequently labeled with ^{3}H-leucine in high [K^+] medium. Proteins into which ^{14}C-leucine was incorporated under normal conditions were exposed to a total of seven hours of high [K^+] treatment, whereas proteins into which ^{3}H-leucine was incorporated under high [K^+] conditions were exposed to four hours or less of high [K^+] treatment. If high [K^+] were affecting only those events which normally occur during or prior to synthesis of the ganglion proteins, and previously synthesized proteins were not affected, then the ^{3}H-labeled pattern should show the usual relative increase over the ^{14}C-labeled pattern at 50K. The ratio pattern from such an experiment should have a peak at 50K, which in fact it did, as shown in Figure 8 (N = 9).

Figure 8. Ratio of ^{3}H (experimental) counts to ^{14}C (control) counts for aqueous soluble fraction of PVG from an internal control experiment in which the experimental media contained 90 mM $[K^{+}]$. (a) Right half of the ganglion. (b) Left half of the ganglion. (See text for explanation). This ganglion was incubated six hours in the experimental incubation medium, instead of the usual four hours. Total counts in gel: (a)^{3}H, 28226 cpm; ^{14}C, 4872 cpm; (b)^{3}H, 24926 cpm; ^{14}C, 12212 cpm.

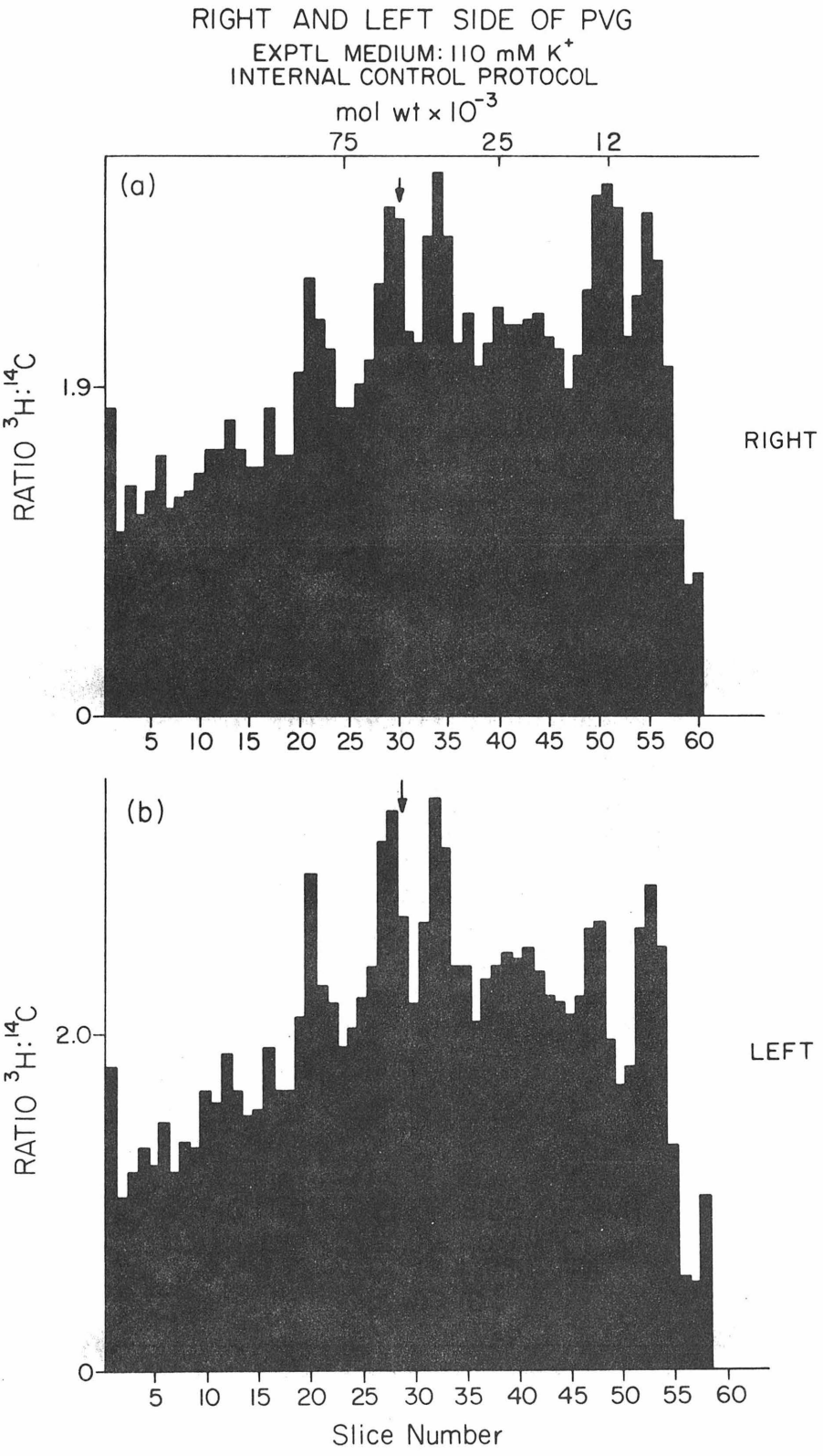

RIGHT AND LEFT SIDE OF PVG
EXPTL MEDIUM: 110 mM K+
INTERNAL CONTROL PROTOCOL
mol wt x 10-3
75
25
12
(a)
RATIO 3H:14C
1.9
0
RIGHT
5 10 15 20 25 30 35 40 45 50 55 60
(b)
RATIO 3H:14C
2.0
0
LEFT
5 10 15 20 25 30 35 40 45 50 55 60
Slice Number

Figure 8 shows two ratio patterns, one for the right half of the ganglion (Figure 8a) and one for the left half of the ganglion (Figure 8b). Since each side of the ganglion contained its own internal control, the ganglion could be cut in half following incubation and the two halves homogenized separately and run on separate gels. The result shows that the high $[K^+]$ effect occurred on both sides of the ganglion.

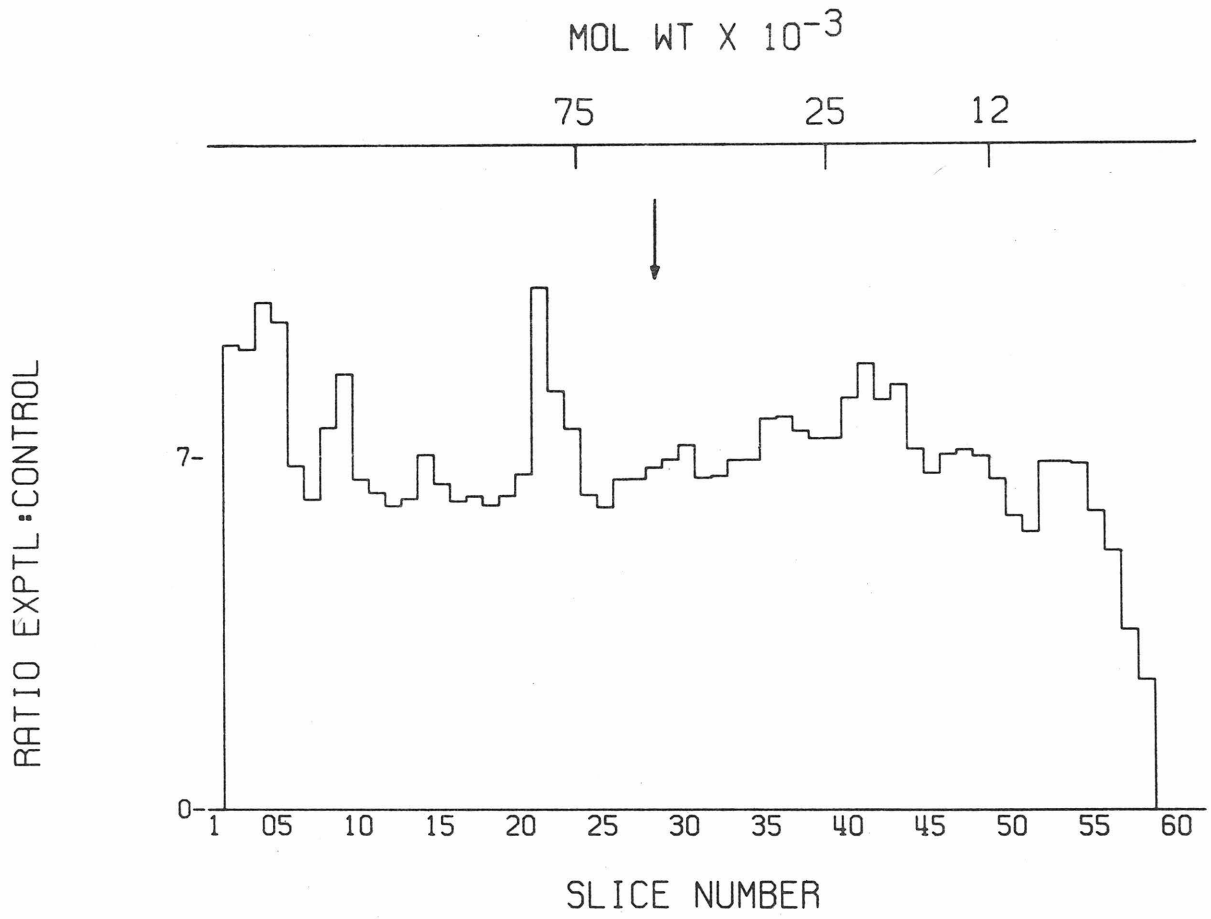
An additional control for the internal control experiment is necessary. Changes in the pattern could be due to the fact that the incubations do not take place at the same time. Thus, the second incubation takes place when the ganglion has been out of the animal longer. Moreover, the earlier incorporated label has had 8 more hours to be "processed" by the ganglion (exported, degraded, etc.) than the later label. Therefore, an additional control for this type of an experiment is a ganglion which has undergone the internal control protocol with normal media during both incubation periods. The ratio pattern for such an experiment shows no peak at 50K (Figure 9, N = 5). Therefore the 50K peak in high $[K^+]$ internal protocol experiments is due to the K^+ and not the time of incubation. In contrast, the peak at 85K, which appears in the ratio pattern for internal control experiments (Figures 8 & 9) and not for parallel control experiments (Figures 3 & 4) seems to be due to the time parameters of incubation. The

Figure 9. Ratio of ^{3}H (experimental) counts to ^{14}C (control) counts for aqueous soluble fraction of PVG from an internal control experiment in which the experimental media and the control media both contained 10 mM [K^{+}]. Total counts in gel: ^{3}H, 52532 cpm; ^{14}C, 7512 cpm.

PARIETO-VISCERAL GANGLION, AQUEOUS SOLUBLE PROTEIN

EXPTL MEDIUM: NORMAL K^+

INTERNAL CONTROL PROTOCOL



85K peak appears in the ratio pattern for internal control experiments regardless of whether the second incubation is in high $[K^+]$ or normal media.

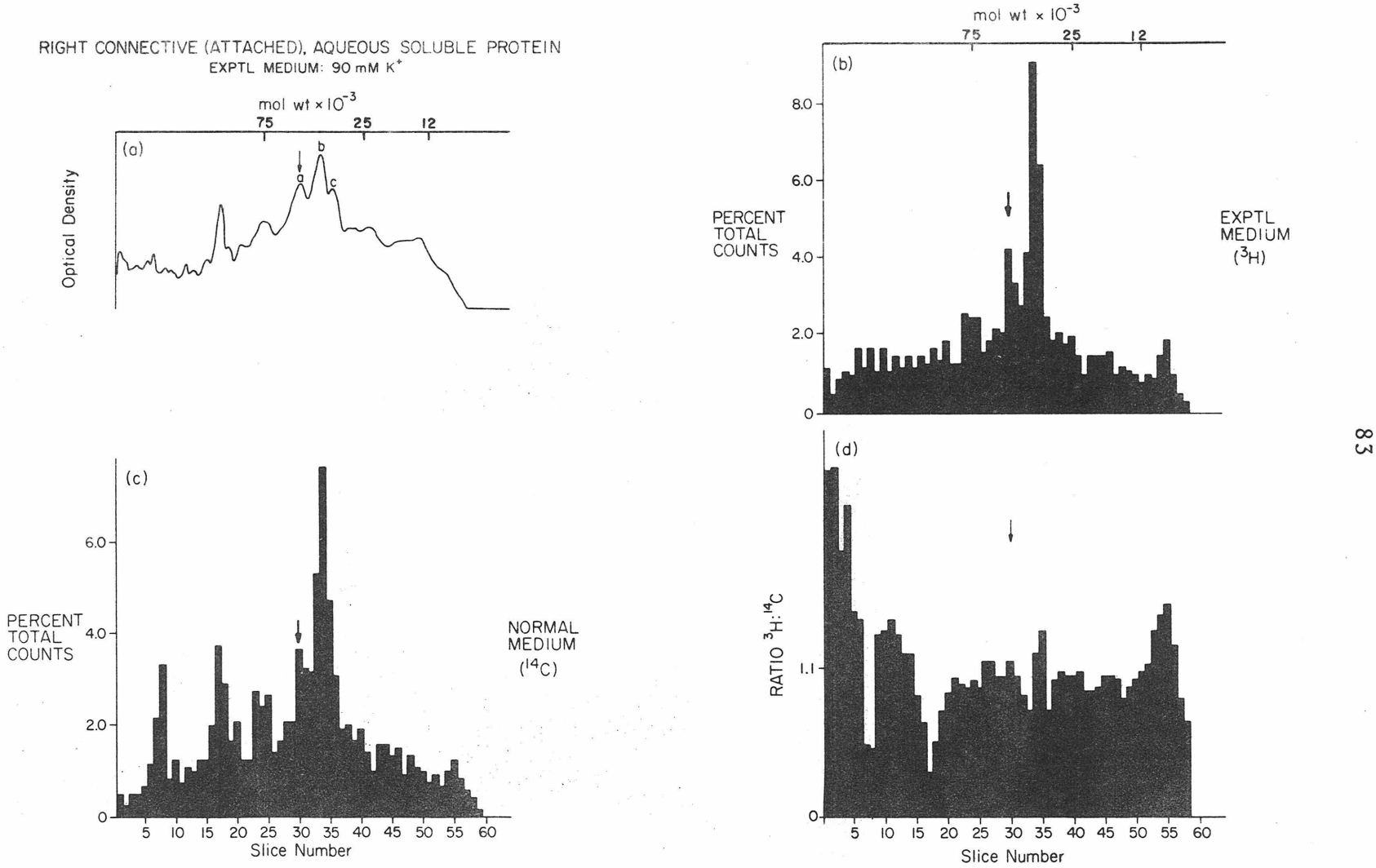
5. Effect of high $[K^+]$ on right connective nerve

It seemed possible that the relative increase in 50K synthesis might be occurring in glial or connective tissue. The presence of the long pleuro-visceral connective nerves (PVCN) joining the PVG to the anterior ganglia provided an easy, though indirect check on this possibility. These nerves contain axons, glia, and connective tissue but no nerve cell bodies.

About one centimeter of the right PVCN located about two centimeters from the control and experimental ganglia was cut free following incubation and then prepared for electrophoresis in a manner similar to preparation of ganglion samples. The result of such an experiment is shown in Figure 10 (N = 5).

Although the staining pattern of the nerve (Figure 10a) is very similar in the 75K to 25K region to that of the ganglion (Figure 2a), the incorporation pattern (Figure 10c) is notably different in that incorporation at 50K is comparatively much smaller. The ratio bar graph (Figure 10d) does not show a large peak at 50K, thereby suggesting that the presence of neuronal cell bodies is necessary to see the high $[K^+]$ effect at 50K.

Figure 10. Patterns of aqueous soluble fraction of right pleuro-visceral connective nerve in a parallel control experiment in which the ^{3}H pattern was from a nerve incubated in experimental media (90 mM $[K^{+}]$, and the ^{14}C pattern was from a nerve incubated in normal media. (a) Staining pattern. Bands a, b, and c are approximately 50K, 40K, and 35K respectively. (b) ^{3}H incorporation pattern. (c) ^{14}C incorporation pattern. (d) Ratio of ^{3}H counts to ^{14}C counts in each slice. Total counts in gel: ^{3}H, 7215 cpm; ^{14}C, 6741 cpm.


RIGHT CONNECTIVE (ATTACHED), AQUEOUS SOLUBLE PROTEIN
EXPTL MEDIUM: 90 mM K+
mol wt x 10-3
75
25
12
(a)
Optical Density
a
b
c
(b)
PERCENT TOTAL COUNTS
EXPTL MEDIUM (3H)
8.0
6.0
4.0
2.0
0
(c)
PERCENT TOTAL COUNTS
NORMAL MEDIUM (14C)
6.0
4.0
2.0
0
5 10 15 20 25 30 35 40 45 50 55 60
Slice Number
(d)
RATIO 3H:14C
1.1
0
5 10 15 20 25 30 35 40 45 50 55 60
Slice Number

A statistical summary of the experiments described above is given in Table V.

6. Effects of high $[K^+]$ on total incorporation

By summing the total number of experimental counts and the total number of control counts on a gel and taking the ratio of these totals, the effect of the experimental conditions on the total amount of incorporation can be calculated. The ratio of experimental to control counts in a parallel control experiment in which both PVG's were in normal media was 8.9 ± 3.5 (N = 9), reflecting the higher specific activity of the label used in the experimental medium. This ratio fell to 4.4 ± 1.0 in 90 mM $[K^+]$ experiments (N = 6); in other words, total incorporation into the aqueous soluble fraction of the ganglion fell to about 50% of the control (significantly different from control at $p < .001$, Mann-Whitney U test and $p < .005$, Student's t test for samples with unequal variances (Bliss, 1967)). The ratio of experimental to control counts in "low $[Na^+]$, normal $[K^+]$" experiments was 9.9 ± 2.2 (N = 3).

A similar calculation can also be done for internal control experiments. When the second incubation period was in normal medium, the average ratio of experimental to control counts was 5.6 ± 1.9 (N = 5). This figure was significantly less than 8.9 ± 3.5 found in parallel control experiments ($p<.05$, Mann-Whitney U Test, $p < .05$, Student's

TABLE V.

SIZE OF 50K PEAK ON RATIO BAR GRAPHS[1]

Type of incubation	$[K^+]$ in exptl. media (mM)	Type of[2] sample	Peak size ± S.D.	N	p (on t test)[3]
Sim. inc.	10	PVG-sol	.98 ± .02	3	n.s.
Sim. inc.	90	PVG-sol	1.01	1	
Parallel	10	PVG-sol	1.05 ± .14	9	
Parallel	90-110	PVG-sol	1.50 ± .15	10	p<.01
Parallel	50	PVG-sol	1.00 ± .13	3	n.s.
Parallel	Reduced Na^+	PVG-sol	.90 ± .01	3	p<.01
Parallel	90	RC-sol	1.11 ± .13	5	n.s.
Parallel	90	PVG-insol	1.06 ± .06	5	n.s.
Parallel	10	PVG-insol	1.08,.90	2	n.s.
Internal	10	PVG-sol	1.01 ± .11	5	
Internal	90-110	PVG-sol	1.38 ± .13	9	p<.01

[1]The size of the peak was obtained as described in Materials and Methods

[2]Abbreviations have the following meanings: PVG, parieto-visceral ganglion; RC, right pleuro-visceral connective nerve; sol, aqueous soluble fraction; insol, aqueous insoluble fraction; sim. inc., simultaneous incorporation; and reduced Na^+, $[Na^+]$ reduced by 80 mM and replaced with 80 mM tris-Cl (pH 7.7). Data for "Internal, 90-110, PVG-sol" include one PVG in which the second labeling was six hours long, instead of the usual four hours.

[3]Student's t test. Where variances were significantly different ($p<.05$ on an F test), an appropriate modification of the t test was used (Bliss, 1967). Probabilities are one-tailed. "n.s." means not significant ($p>.05$).

t test, and probably reflected reduced incorporation rates during the second incubation period as well as continued incorporation of residual label from the first incubation period. In high $[K^+]$ experiments, the average ratio fell to 2.5 ± .8 (N = 8), or 45% of the normal ratio for this protocol ($p < .001$, Mann-Whitney U test, $p < .005$, Student's t test).

In parallel control experiments on the right PVCN, 90 mM $[K^+]$ caused the ratio of experimental to control counts to fall to 1.2 ± .8 (N = 5), i.e. only about 13% of the control value determined in the PVG. As a check on this remarkable degree of inhibition, the specific activity per unit protein obtained in nerve from normal and high $[K^+]$ incubations was measured.

Dissected lengths of right or left PVCN were pre-incubated for 3 hours and incubated for 4 hours in normal or experimental media, using ^{3}H-leucine as the label in all cases. (Note: This differed from gel electrophoresis studies, in which PVCNs remained attached to PVGs until the end of incubation. The procedure used here for specific activity experiments enabled intra-animal comparison of nerves incubated in different media.) Incubation was followed by several rinses in normal or experimental blood salts, respectively, at 0^oC, to wash off easily exchangeable leucine in the extracellular space. Non-volatile TCA soluble radioactivity and specific activity of proteins in

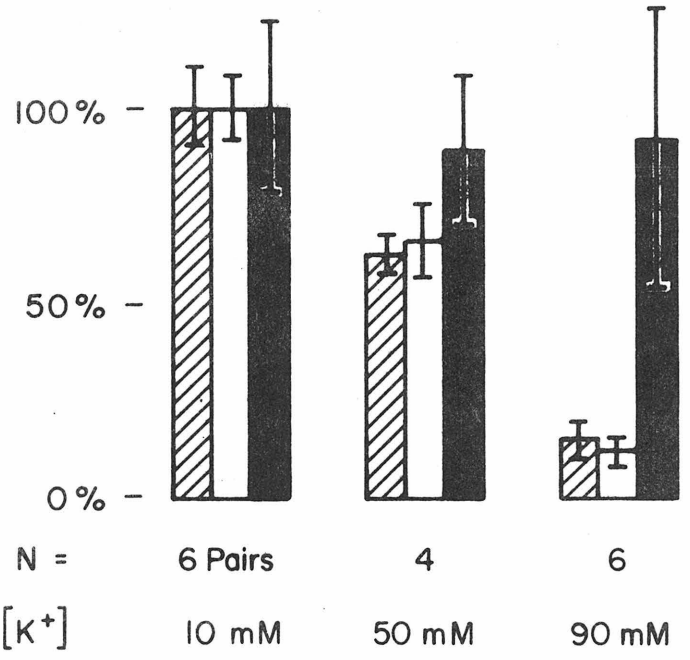
the aqueous soluble fraction and the aqueous insoluble pellet were determined. TCA soluble radioactivity and specific activities of protein from nerves incubated in experimental media were expressed as percent of the mean of two pieces of nerve from the same animal incubated in normal media. The results are shown in Figure 11.

Ninety mM $[K^+]$ reduced incorporation into protein to about 15% of the normal level in the aqueous soluble fraction ($N = 6$, $p < 10^{-4}$, t test for paired variates), in agreement with the value determined by comparing total counts found in gels. The aqueous insoluble fraction was similarly affected. In contrast, the average amount of non-volatile TCA soluble label found in the nerves was not significantly reduced by high $[K^+]$ treatment. Fifty mM $[K^+]$ caused a more moderate decrease in incorporation into protein than 90 mM $[K^+]$ and likewise did not significantly reduce TCA soluble counts in the nerve ($N = 4$). When the specific activity of protein in the PVG was similarly measured under normal ($N = 3$) and high $[K^+]$ ($N = 3$) conditions, the results confirmed the 50% inhibition previously calculated from gel electrophoresis results. Non-volatile TCA soluble counts in the ganglion did not change. (Table VI).

To test whether the connective tissue sheath of the ganglion was affected by high $[K^+]$ to as great a degree as were nerves, ganglia were incubated according to the

Figure 11. Short pieces of PVCN were incubated in normal media (10 mM $[K^+]$ or media containing 50 mM or 90 mM $[K^+]$, according to the protocol described in the text. Levels of incorporation are expressed as percent of the mean incorporation of two pieces of nerve from the same animal incubated in normal media. The levels of incorporation in normal media, averaged over all animals (N = 6), were the following: aqueous soluble, TCA precipitable, $1.36 \pm .46 \times 10^6$ cpm/mg protein; aqueous insoluble, $1.22 \pm .34 \times 10^6$ cpm/mg protein; and TCA soluble, non-volatile, $6 \pm 4 \times 10^7$ cpm/mg aqueous soluble protein. These experiments were performed in December, 1972 and April, 1973. Specific activities of all three fractions were lower (by about 1/3) in April than December, but the magnitude of the high $[K^+]$ effect at both times was the same.

EFFECT OF $[K^+]$ ON INCORPORATION OF ^3H-LEUCINE INTO PLEURO-VISCERAL CONNECTIVE NERVE



▨ aqueous soluble, TCA precipitable (cpm/mg protein)

□ aqueous insoluble (cpm/mg protein)

■ TCA soluble, non-volatile (cpm/mg aqueous soluble protein)

TABLE VI.

EFFECT OF $[K^+]$ ON INCORPORATION OF ^{3}H-LEUCINE

INTO PARIETO-VISCERAL GANGLION

	$[K^+]$		$\frac{90\ mM}{10\ mM}$
	10 mM	90 mM	
Aqueous soluble fraction			
TCA insoluble (cpm/mg protein)	$1.24 \pm .44 \times 10^6$	$.62 \pm .26 \times 10^6$	50%[1]
TCA soluble $\left[\frac{\text{non-volatile cpm}}{\text{mg aqueous soluble protein}}\right]$	$8.5 \pm 6 \times 10^6$	$8.5 \pm 4 \times 10^6$	100%
Aqueous insoluble pellet (cpm/mg protein)	$1.31 \pm .41 \times 10^6$	$.68 \pm .33 \times 10^6$	53%[1]

[1]significantly different from 100% at p = .05 (Student's t test).

From the known specific activity (50 μCi/1.05 μg leucine) and counting efficiency (about 40%) of ^{3}H-leucine, one can calculate the amount of leucine incorporated per hour. 1.3×10^6 cpm/mg = 1.3×10^6 cpm/mg x 1 dpm/.4 cpm x 1 μCi/2.22×10^6 dpm x 1.05 μg leucine/50 μCi ÷ 4 hr = .008 μg leucine/mg protein/hr. This is about half the rate seen by Wilson (1971) in dissected R2 cells; however, due to the large variances the difference is not significant. This experiment was done in April, 1973.

internal control protocol. At the end of the second incubation period, ganglia were rinsed briefly in blood salts media at $0^{o}C$. Ganglia were then slit open and neuronal and neuropil constituents, including the bag cells were scraped free from the connective tissue sheath (CTS). The ratio of experimental to control counts in the TCA precipitable fraction of the connective tissue sheath and the neuronal/neuropil regions of the ganglion were measured. High $[K^{+}]$ caused only slightly more inhibition in the connective tissue sheath than in the neuronal/neuropil region. Incorporation averaged 43% of normal in connective tissue sheath and 65% of normal in the neuronal/neuropil region (N = 3 normal and 3 high $[K^{+}]$ experiments. See Table VII). The neuronal/neuropil region accounted for 62 $\pm$ 17% of the incorporation into the aqueous soluble protein of the ganglion (N = 6, data from the control counts for these internal control experiments), in agreement with the value determined by Schwartz, et al. (1971) for incorporation into total protein of the ganglion. This experiment is marred by large variances and the fact that the ratio of experimental to control counts for both normal and high $[K^{+}]$ incubations was lower than for previous internal control experiments.

7. Effects of high $[K^{+}]$ incubation on the solubility of labeled protein

TABLE VII.

EFFECT OF $[K^+]$ ON INCORPORATION INTO

CONNECTIVE TISSUE SHEATH AND NEURONAL/NEUROPIL REGION

Tissue	Connective tissue sheath		Neuronal/neuropil region	
$[K^+]$ in experimental media	10 mM	90 mM	10 mM	90 mM
Ratio of experimental to control counts	3.0	1.3	2.6	1.7
±	±	±	±	±
standard deviation	.9	.9	1.0	.5
(N)	(3)	(3)	(3)	(3)
$\frac{10\ mM}{90\ mM}$	43%[1]		65%[2]	

[1] $p < .05$, Student's t test

[2] not significantly different from 100%

The relative increase in incorporation at 50K in aqueous soluble proteins might be interpreted as a relative increase in 50K peptide(s) being synthesized. An alternative would be that high $[K^+]$ makes most other peptides in this fraction relatively less soluble. In order to check this, the percentage of labeled protein which was aqueous soluble was determined. PVGs were labeled and homogenized according to a parallel control protocol as usual, using 90 mM $[K^+]$ for the experimental ganglion. The homogenate in .001 M phosphate buffer was then processed as shown in Figure 12 in order to determine the relative distribution of label into the aqueous insoluble pellet (P1 in Figure 12) and the aqueous soluble, TCA insoluble pellet. Results are shown in Table VIII.

For ganglia incubated in high $[K^+]$ about 32% of the TCA precipitable label in the ganglia was aqueous soluble. Ganglia incubated in normal $[K^+]$ had about 37% of their TCA precipitable label in the aqueous soluble fraction ($p < .03$, t test for paired variates). The possible role of this change in relative amount of label in the aqueous soluble fraction in producing the change at 50K will be considered further in the discussion.

DISCUSSION

High potassium media ($[K^+]$ = 90-110 mM, with an equimolar reduction of $[Na^+]$) caused a relative increase in

Figure 12. Schematic diagram of the method of preparation of samples for determining the distribution of TCA insoluble radioactivity. All pellets were counted in 15 ml of counting cocktail containing NCS solubilizer (Ward, et al., 1970).

PREPARATION OF SAMPLES
FOR DETERMINATION OF SOLUBILITY
OF LABELED PROTEIN

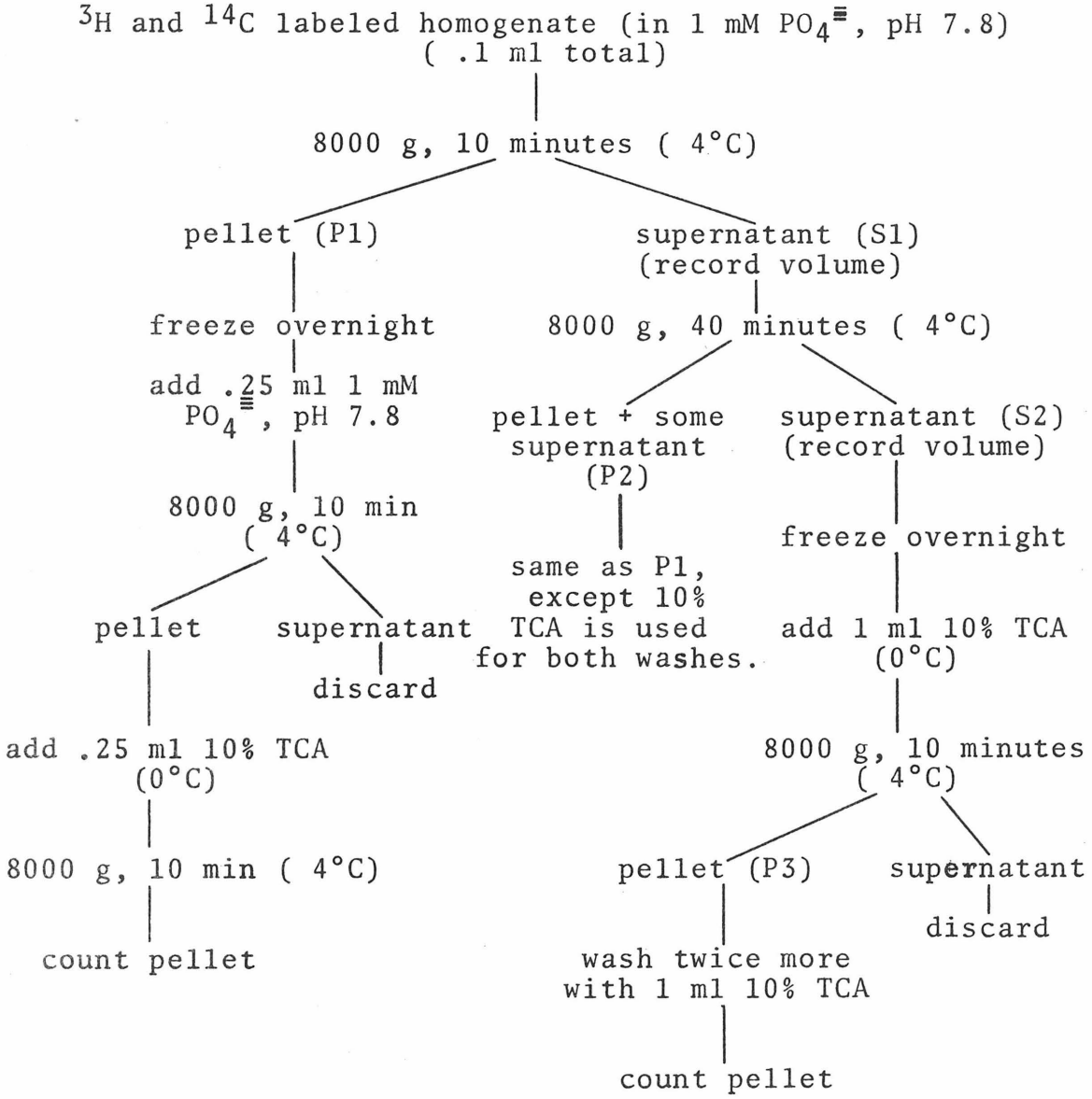


FIGURE 12

TABLE VIII. DISTRIBUTION OF

LABEL IN TCA INSOLUBLE FRACTIONS

Fraction			P1 aqueous insoluble	P2 mostly aqueous[1] soluble	P3[2] aqueous soluble	$^3H, ^{14}C$ P3	$^3H, ^{14}C$ P2+P3
Expt.	Label	$[K^+]$ (mM)					
1	^3H	90	65.8%	3.6%	30.6%	2.2%	2.6%
	^{14}C	10	63.2	4.0	32.8		
2	^3H	90	69.0	2.3	28.7	7.0	6.9
	^{14}C	10	62.1	2.2	35.7		
3	^3H	90	69.8	4.4	25.8	5.3	5.6
	^{14}C	10	64.2	4.7	31.1		
				P2 + P3			
MEAN ± S.D.		^3H		31.6 ± 1.8		4.8 ± 2.5	5.0 ± 2.1[3]
		^{14}C		36.8 ± 1.0			

[1]P2 includes TCA precipitable counts of P3 which were left in the test tube when the supernatant was removed. This generally amounts to about 10% of whatever is being removed. Therefore, virtually all of the counts in P2 belong in the P3 fraction.

TABLE VIII. (CONTINUED)

[2]Corrected for the difference in volume between S1 and S2 (see Figure 12). The counts found in P3 are multiplied by Volume(S1)/Volume(S2) for comparison with the other pellets.

[3]$p<.03$, t test for paired variates.

incorporation of leucine into aqueous soluble peptides of approximately 50,000 molecular weight in the parieto-visceral ganglion of Aplysia. This change did not occur in the SDS soluble proteins of the aqueous insoluble fraction of the ganglion, nor did it occur in pleuro-visceral connective nerve subjected to similar treatment.

When $[Na^+]$ was lowered without raising $[K^+]$, no relative increase at 50K occurred, suggesting that the "high $[K^+]$" effect was indeed due to increased $[K^+]$, and not reduced $[Na^+]$. Alternative interpretations are that both low $[Na^+]$ and high $[K^+]$ are required for the effect, or that $tris^+$, which was added instead of K^+, counteracts a low $[Na^+]$ effect. The high $[K^+]$ interpretation is favored mostly on the basis of parsimony. The effects of high $[K^+]$ on other phenomena (total incorporation into protein (Jones & McIlwain, 1971; Folbergrova, 1961; Mase, et al., 1962) and respiration (Mase, et al.)) has been studied with cumulatively more extensive controls for $[Na^+]$ effects, and at least with these phenomena, the effects seem to be due to $[K^+]$.

Internal control experiments showed that high $[K^+]$ probably does not increase the relative incorporation at 50K by affecting previously synthesized proteins. Proteins which were synthesized while the PVG was in normal media showed a smaller 50K peak than proteins synthesized in a subsequent high $[K^+]$ incubation. The only "post-synthesis

interpretation" compatible with this result is one in which proteins are susceptible to high $[K^+]$ effects only for a few hours after synthesis.

The relative increase at 50K is also unlikely to be due to the proteins located at other molecular weights becoming relatively less soluble. Data in section 7 of the results show that the proteins into which label is incorporated in high $[K^+]$ are less soluble than those labeled in normal media. For the 50K peak to increase in relative size by 40%, as it has (Table III), the amount of label in other proteins would have to decrease by 30%. This would mean a decrease in the percentage of aqueous soluble, TCA insoluble label from 37% to 25%, a change much larger than has been observed. The smaller change observed may be a non-specific decrease in solubility of proteins, or it may represent an increase in relative incorporation into one or a few aqueous insoluble proteins.

These results raised the possibility that the patterns of aqueous soluble proteins synthesized by neurons in the ganglion were qualitatively changed in high $[K^+]$ media. To test this possibility more directly, aqueous soluble patterns of the giant cell of the PVG have been studied using the internal control method (Chapter III). The incorporation patterns obtained were exceedingly variable, particularly in the 50K region; however, the internal control made some analysis possible. In high $[K^+]$

experiments where the 50K peak was present in the control pattern (N = 3), a relative increase in incorporation at 50K occurred. In normal $[K^+]$ experiments where the 50K peak was present in the control pattern (N = 2), no such increase was observed. Since the source of the variability in the control patterns is unknown, this is only weak supporting evidence that high $[K^+]$ caused changes in individual neurons.

An alternative hypothesis to be considered was that individual cell patterns remained qualitatively the same but the relative contribution of cells having different patterns changed, viz.: cells synthesizing little 50K protein might drastically decrease their synthesis relative to cells synthesizing a lot of 50K protein. There was, however, no evidence that large changes in the relative contribution of different populations of cells in the ganglion occurred in high $[K^+]$ experiments. No significant difference in the degree of inhibition of total incorporation into connective tissue and in the neuronal/neuropil regions of the ganglion was seen (section 6). Incorporation into the giant cell in high $[K^+]$ experiments averaged 65% of normal (N = 6 pairs, Chapter III), and was similar to that found in the whole ganglion. Autoradiography suggests that the glia probably constitute less than 20% of the total incorporation into the neuronal/neuropil regions of the ganglion (Strumwasser, 1967 and Chapter IV). Even if glial

incorporation were drastically reduced compared to neuronal incorporation, it probably constitutes too small a fraction of the total synthesis to cause a relative change of the magnitude seen at 50K. It seems most likely that changes in the whole ganglion reflect qualitative changes taking place in individual cells of the ganglion, though the evidence for this is weak and indirect. More convincing experiments at the single cell level are necessary.

No experiments have been done yet to find out whether the depolarization caused by high $[K^+]$ or some other effect of $[K^+]$ caused the changes observed in the ganglion. In the giant cell, it is known that high $[K^+]$ causes large increases of intracellular $[Cl^-]$ (Russell & Brown, 1971). High $[K^+]$ also causes transmitter and neurohormone (Arch, 1972) release in the ganglion. High $[K^+]$ should affect energy requiring ion transport as well, and thus could well cause changes by altering normal energy flow in the cells (Heald, 1960). Depolarizing the ganglion with high $[K^+]$ while some of these other processes are blocked (low $[Ca^{++}]$, high $[Mg^{++}]$ to block transmitter and neurohormone release; ouabain to block ion transport) could be a way of testing some of the alternatives.

The experiments reported here also do not satisfactorily resolve the question of whether we are actually seeing a change in synthesis of proteins or merely a change in incorporation. The latter could occur without the

former if, for example, transport of the label into the pool from which the 50K protein is being synthesized was affected differently by high $[K^+]$ than was transport into pools for other proteins. This is a very difficult point to check since any heterogeneous piece of tissue contains many pools. No change occurred in the amount of non-volatile TCA soluble label in the PVG or connective nerve in 90 mM $[K^+]$ despite decreases in incorporation. It can be argued, however, that much of the label resided in pools which were inactive in protein synthesis. A further consideration, not experimentally investigated, is that some of this TCA soluble label may be in leucine metabolites, the relative amounts of which may change.

The 7-fold decrease in incorporation into nerve is a rather remarkable result. This degree of inhibition by high $[K^+]$ media is larger than most previously reported effects of $[K^+]$ on protein synthesis in nervous tissue. Further experiments on this effect have been done and are reported elsewhere (Chapter V). These experiments show that the inhibition takes place in both the connective tissue sheath and the axonal (presumably glial synthesis) region of the nerve.

It was noted earlier that the staining pattern and the incorporation pattern of the PVG differed at several points. Thus, the largest staining band is second largest in incorporation; the second largest staining band is

largest in incorporation; and the third largest staining band is a trough in the incorporation pattern. Such differences might be expected if labeled proteins were exported from the ganglion, via neurosecretion or axonal transport. These differences also might be due to differences in rate of turnover of different proteins. Differences in leucine incorporation could also reflect differences in the fractional amount of leucine in different proteins.

Differences between staining and incorporation patterns may also reflect perturbations of amino acid incorporation brought about by dissection of the PVG from the animal and the *in vitro* incubation. As described in the section on the *internal control* experiment, changes in which proteins were labeled was partially a function of the timing of incubations after dissection of the ganglion from the animal. It is not known whether either of these patterns is the same as would be found if the incorporation were done *in vivo*. Wilson (1971), using a less sensitive technique of comparison, reported that *in vivo* patterns of single cells did not differ significantly from their *in vitro* patterns. The changes between patterns obtained from the first and second incubations of *internal control* experiments having normal media present throughout (Figure 9) may represent changes taking place in protein synthesis due to the *in vitro* method of incubation.

The staining pattern of the nerve showed a peak at

50K (Figure 10a), yet the nerve did not synthesize much 50K material (Figure 10c). In contrast, the ganglion showed relatively more synthesis of 50K material than did its staining pattern (Figures 2a & 2c). Although other hypotheses are possible, the 50K material may be synthesized in the ganglion and transported into the nerve. The 50K peptide may correspond to tubulin subunits, found in microtubles (Borisy & Taylor, 1967; Shelanski & Taylor, 1967), which may be involved in axonal transport (Kreutzberg, 1969; Barondes & Feit, 1969). One may speculate whether the 50K peptide stained in the nerve corresponds to the 50K peptide into which incorporation is relatively increased in the ganglion. No experiments have been done to test the identities or functions of these 50K peptides.

SUMMARY

The effect of high $[K^+]$ media, which cause prolonged depolarization of neurons and glial cells, on the incorporation of leucine into proteins of the parieto-visceral ganglion (PVG) and pleuro-visceral connective nerves (PVCN) of Aplysia californica was studied. PVGs were rinsed in artificial blood salts media for three hours and then incubated in a defined medium containing ^{3}H- or ^{14}C-leucine for four hours. To study the effects of $[K^+]$, $[K^+]$ was raised in both the rinse period and the incubation period. Incorporation of labeled leucine into peptides of

different molecular weights was examined on sodium dodecyl sulfate polyarylamide gels.

High $[K^+]$ (90-110 mM, with equimolar $[Na^+]$ reduction) caused a relative increase of incorporation at 50K (K = 1000 daltons) and 40K in the aqueous soluble fraction of the PVG. The larger effect, at 50K, was examined further.

(1) A relative increase at 50K due to high $[K^+]$ did not occur in the SDS soluble proteins of the aqueous insoluble fraction of the PVG. Therefore, the effect is not a general one on all 50K peptides in the ganglion.

(2) The effect did not occur when $[K^+]$ was raised to only 50 mM.

(3) A relative increase at 50K did not occur in PVCNs cut from PVGs incubated in high $[K^+]$. PVCNs contain connective tissue, glial cells, and axons but no nerve cell bodies. It therefore seems likely that the effect requires the presence of nerve cell bodies, as in the ganglion.

(4) A relative increase at 50K did not occur when $[Na^+]$ was lowered and $tris^+$ was added instead of K^+. The most parsimonious interpretation of this result is that the "high $[K^+]$" effect is unlikely to be a "low $[Na^+]$" effect.

(5) PVGs initially labeled with ^{14}C-leucine in normal medium and then subsequently labeled with ^{3}H-leucine in high $[K^+]$ medium showed relatively more ^{3}H label at 50K than ^{14}C label. Therefore, the high $[K^+]$ effect is unlikely to

be a post-synthesis effect.

High [K^+] also caused a decrease in total incorporation into the PVG and the PVCN. Total incorporation into PVG was decreased by 50%, and total incorporation into PVCN was decreased by 85%. Incubation in high [K^+] caused a decrease in the percent of TCA insoluble label in the PVG which was aqueous soluble; however, this decrease was too small to attribute the relative increase at 50K to a decreased solubility of peptides located at other molecular weights.

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CHAPTER III

LEUCINE INCORPORATION INTO THE GIANT CELL OF *APLYSIA CALIFORNICA*:

1. MOLECULAR WEIGHT DISTRIBUTIONS OBTAINED FROM AQUEOUS SOLUBLE AND AQUEOUS INSOLUBLE FRACTIONS
2. EFFECTS OF HIGH K^+ MEDIA.

Much is known about the biochemical and electrophysiological properties of the giant cell (cell R2 in the nomenclature of Frazier, et al., 1967) of the parietovisceral ganglion (PVG, or abdominal ganglion) of *Aplysia californica*. DNA (Lasek & Dower, 1971), RNA (many papers, including Wilson & Berry, 1972), transmitter related enzymes (Giller & Schwartz, 1971a,b; McCaman & Dewhurst, 1970), and proteins, the topic of this chapter, have been investigated. Wilson (1971) described the pattern seen on SDS polyacrylamide gels of labeled leucine incorporated into proteins of the giant cell. He contrasted the giant cell pattern to that found in other cells of the ganglion, and later (Wilson & Berry, 1972) looked for effects of synaptic stimulation of the giant cell on its incorporation pattern. No effects of synaptic stimulation were seen.

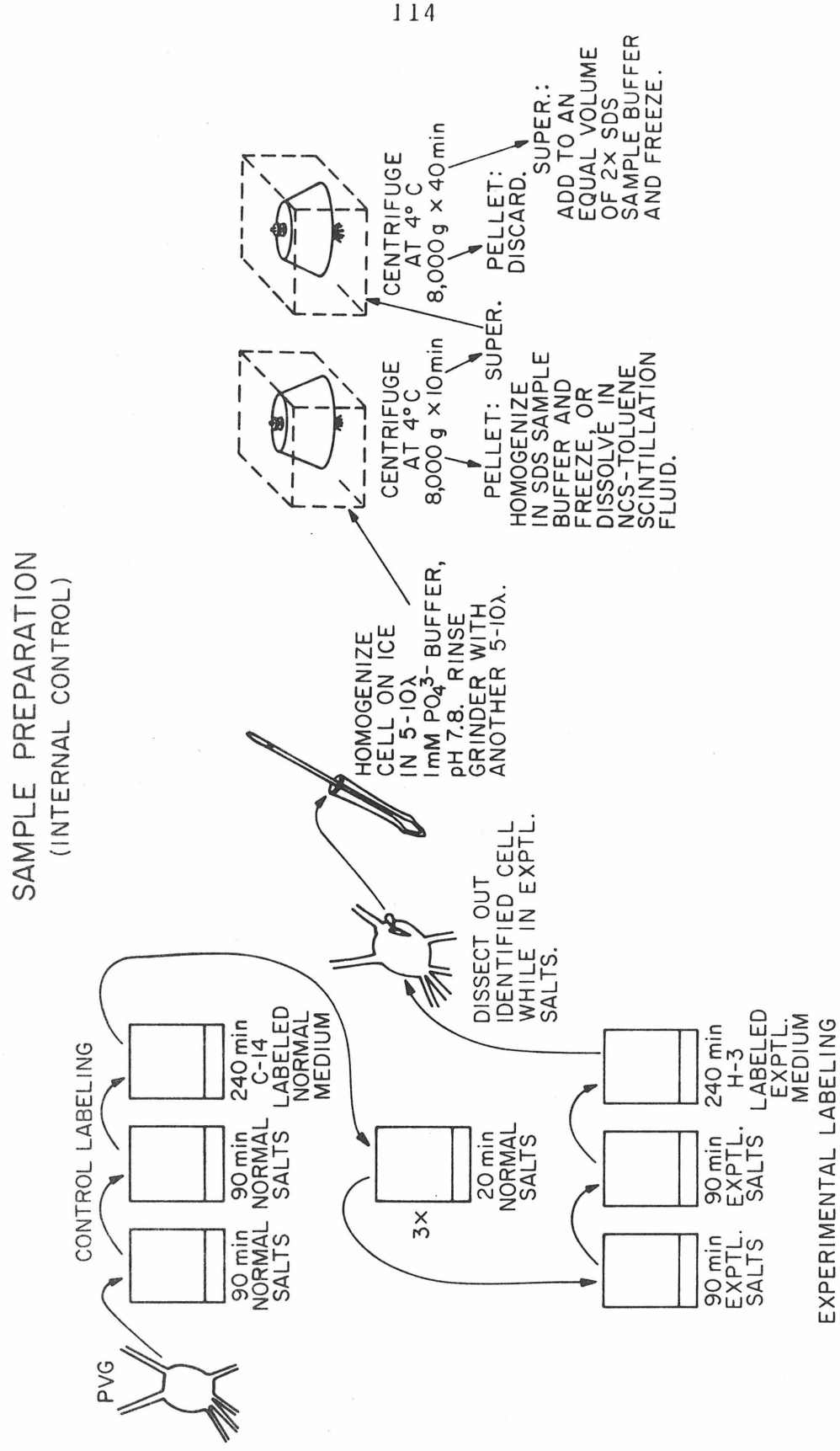
In the previous chapter I showed that incubation in high $[K^+]$ media caused a change in the incorporation pattern of leucine into the aqueous soluble fraction of the

PVG. To investigate whether this effect was occurring at the level of single neurons, an investigation of the effect of high $[K^+]$ incubation on the giant cell pattern was begun. This chapter extends Wilson's studies on the molecular weight distribution of proteins synthesized in the giant cell to the aqueous soluble and aqueous insoluble fractions. Under the conditions used, the aqueous soluble fraction was very variable; however, the use of an internal control method enabled the demonstration of high $[K^+]$ effects similar to those found in the whole ganglion.

MATERIALS AND METHODS

PVGs were dissected as previously described (Chapter II) from adult Aplysia californica, maintained at 13-14° C in a filtered sea water system. The incubation procedure was of the internal control type, used in some of the previously described experiments (Chapter II). This protocol is shown schematically in Figure 1: The PVG went through two 90 minute rinses in normal blood salts medium; a 240 minute incubation in a normal incubation medium, which contained ^{14}C-leucine; three 20 minute rinses in a normal blood salts medium, to wash out as much of the ^{14}C label as possible; two 90 minute rinses in experimental blood salts medium; a 240 minute incubation in an experimental incubation medium, which contained ^{3}H-leucine; and finally a variable length rinse in experimental blood salts

Figure 1. Schematic diagram of internal control incubation protocol and sample preparation.


SAMPLE PREPARATION
(INTERNAL CONTROL)
PVG
CONTROL LABELING
90 min NORMAL SALTS
90 min NORMAL SALTS
240 min C-14 LABELED NORMAL MEDIUM
3×
20 min NORMAL SALTS
DISSECT OUT IDENTIFIED CELL WHILE IN EXPTL. SALTS.
HOMOGENIZE CELL ON ICE IN 5-10λ 1mM PO_4^{3-} BUFFER, pH 7.8. RINSE GRINDER WITH ANOTHER 5-10λ.
CENTRIFUGE AT 4° C 8,000 g × 10 min
PELLET: SUPER.
HOMOGENIZE IN SDS SAMPLE BUFFER AND FREEZE, OR DISSOLVE IN NCS-TOLUENE SCINTILLATION FLUID.
CENTRIFUGE AT 4° C 8,000 g × 40 min
PELLET: DISCARD.
SUPER.: ADD TO AN EQUAL VOLUME OF 2× SDS SAMPLE BUFFER AND FREEZE.
90 min EXPTL. SALTS
90 min EXPTL. SALTS
240 min H-3 LABELED EXPTL. MEDIUM
EXPERIMENTAL LABELING

while the giant cell was being dissected. In a few experiments the experimental incubation period was extended (360 minutes, two cells) or shortened (to less than 150 minutes, four cells; in these cases, the leucine used had 8 times higher specific activity than usual). Contents of media are given in Table I, Chapter II.

Giant cells were dissected from the ganglion and homogenized on ice in a small volume (5-10 µl) of 1mM Na-phosphate (pH 7.8). The homogenizer was rinsed with another 5-10 µl of phosphate buffer, and the combined sample was centrifuged at 8000g for 10 minutes (≤4° C). The supernatant was centrifuged again at 8000g for 40 minutes (≤4° C), and the final supernatant, called here the aqueous soluble fraction, was combined with an equal volume of 2X sodium dodecyl sulfate (SDS) sample buffer (Chapter II) and frozen at -10° C for later analysis. The preparation of this "aqueous soluble fraction" is thus identical to that used for the "aqueous soluble fraction" of the ganglion (Chapter II), except that the volumes used here are smaller. The pellet from the first centrifugation was either further homogenized in normal SDS sample buffer (Chapter II) and frozen for later analysis, or it was solubilized in scintillation fluid containing Nuclear Chicago Solubilizer (Ward, et al., 1970) and counted.

The samples were run on SDS polyacrylamide gels, as previously described (Chapter II). After electrophoresis,

gels were stained several hours in 0.25% Coomassie Brilliant Blue in 9% acetic acid, 45% methanol and then destained by diffusion in 7.5% acetic acid, 5% methanol for several days. Usually there was not enough protein on the gel to show protein bands clearly. This staining-destaining procedure removed the free leucine radioactivity which usually runs near the high molecular weight end of the gel, but may have rapidly migrating components as well (Wilson, 1971). The procedure for slicing and counting gels has been described before (Chapter II).

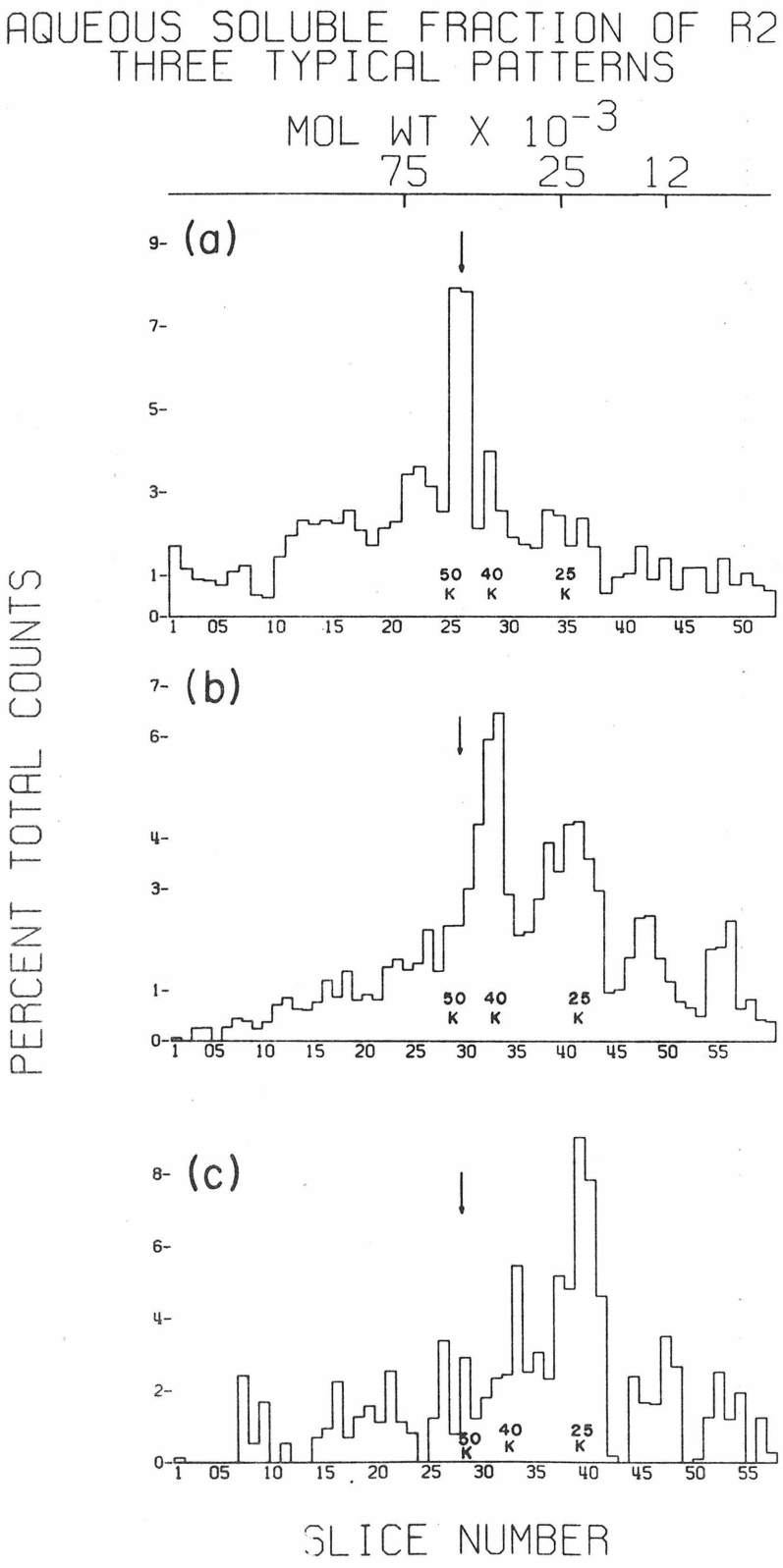
Data were analyzed and plotted on an IBM batch processor. After correcting for background counts and overlap between channels, bar graphs of the control counts, of the experimental counts, and of the ratio of control to experimental counts were plotted. The size of a peak on the ratio bar graph was determined as in the previous chapter (Chapter II).

RESULTS

1. Normal patterns of incorporation

The pattern of incorporation into the aqueous soluble fraction of R2 was very variable in the 35K-75K region. Figure 2 shows three representative patterns observed. The patterns are of ^{14}C labeled peptides into which label was incorporated during the initial normal incubation period.

Figure 2. Three representative gel patterns of the aqueous soluble fraction of R2. These are control patterns of ^{14}C-labeled peptides. Normal [K^+] was present in both the control and experimental phases of the experiments. The positions of 50K, 40K, and 25K (K = 1000 daltons) peptides are indicated. When prominent, 50K and 40K peak positions were determined by examination; otherwise these "peaks" were located relative to the migration of the peak at 25K. Protein molecular weight calibration was determined by average migration relative to the bromphenol blue tracking dye. Total ^{14}C counts in gel: (a) 409 cpm, (b) 548 cpm, (c) 70 cpm.


AQUEOUS SOLUBLE FRACTION OF R2
THREE TYPICAL PATTERNS
MOL WT X 10^{-3}
75
25
12
(a)
(b)
(c)
50 K
40 K
25 K
PERCENT TOTAL COUNTS
SLICE NUMBER

In Figure 2a, there are peaks at 50K, 40K, and 25K. In Figure 2b, only a shoulder on the 40K peak appears where the 50K peak had been, and finally, in Figure 2c, the incorporation at both 50K and 40K has become much reduced compared to the 25K peak. The changes at 50K were of particular interest since changes in incorporation at this molecular weight occurred in ganglia incubated in high $[K^+]$ media. An attempt was made to find some variable that correlated with the percent of total counts in the gel located in two slices at 50K. No correlation with time of year, sexual maturity (weight of reproductive tract), or the second incubation being in high $[K^+]$ was found. There was, however, a correlation with the fraction of counts in the cell which were aqueous soluble protein. This relationship is shown in Figure 3. The relevance of this observation to high $[K^+]$ effects in the ganglion will be considered in the discussion section of this chapter.

Figure 4 shows a typical ^{14}C labeled control pattern obtained when the aqueous insoluble fraction of R2 was run on a gel. The patterns resemble those obtained for total SDS soluble protein by Wilson (1971). The most heavily incorporating peak runs approximately the same distance with respect to the tracking dye as does the 50K peak in the aqueous soluble fraction.

Figure 3. Relation between percent of aqueous soluble protein appearing in two slices located at 50K (% counts at 50K) and percent of counts in the cell which were aqueous soluble protein (% aq. sol. protein).

$$\% \text{ aq. sol. protein} = \frac{AS}{AS+PEL}$$

where AS is the total number of counts on the gel from an aqueous soluble sample, corrected for pipetting losses, and PEL is the number of counts in the aqueous insoluble pellet. ⊙, control patterns of internal control experiments. ⊡, △, patterns from experiments where R2 was dissected at the end of the control labeling period.

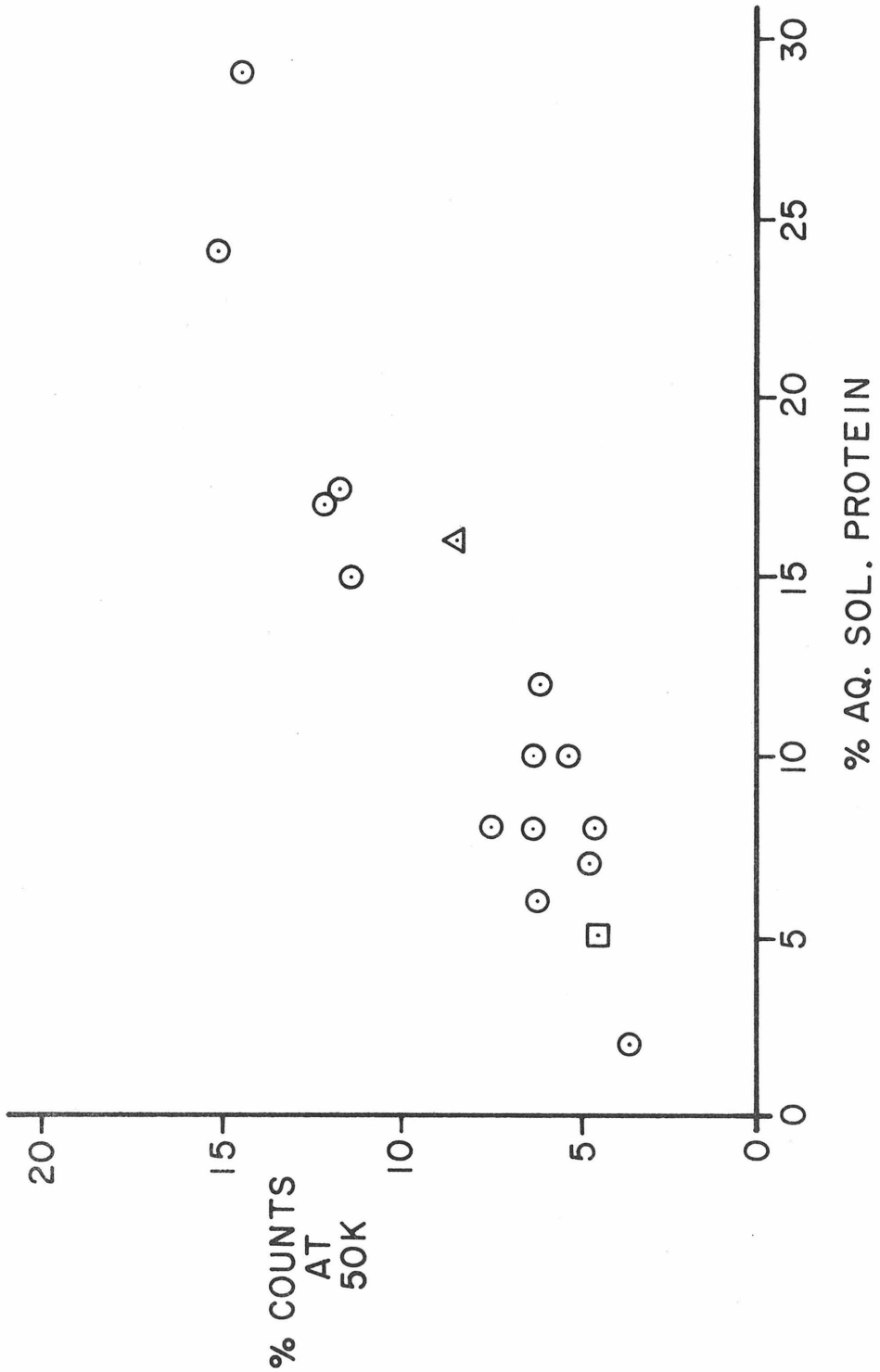
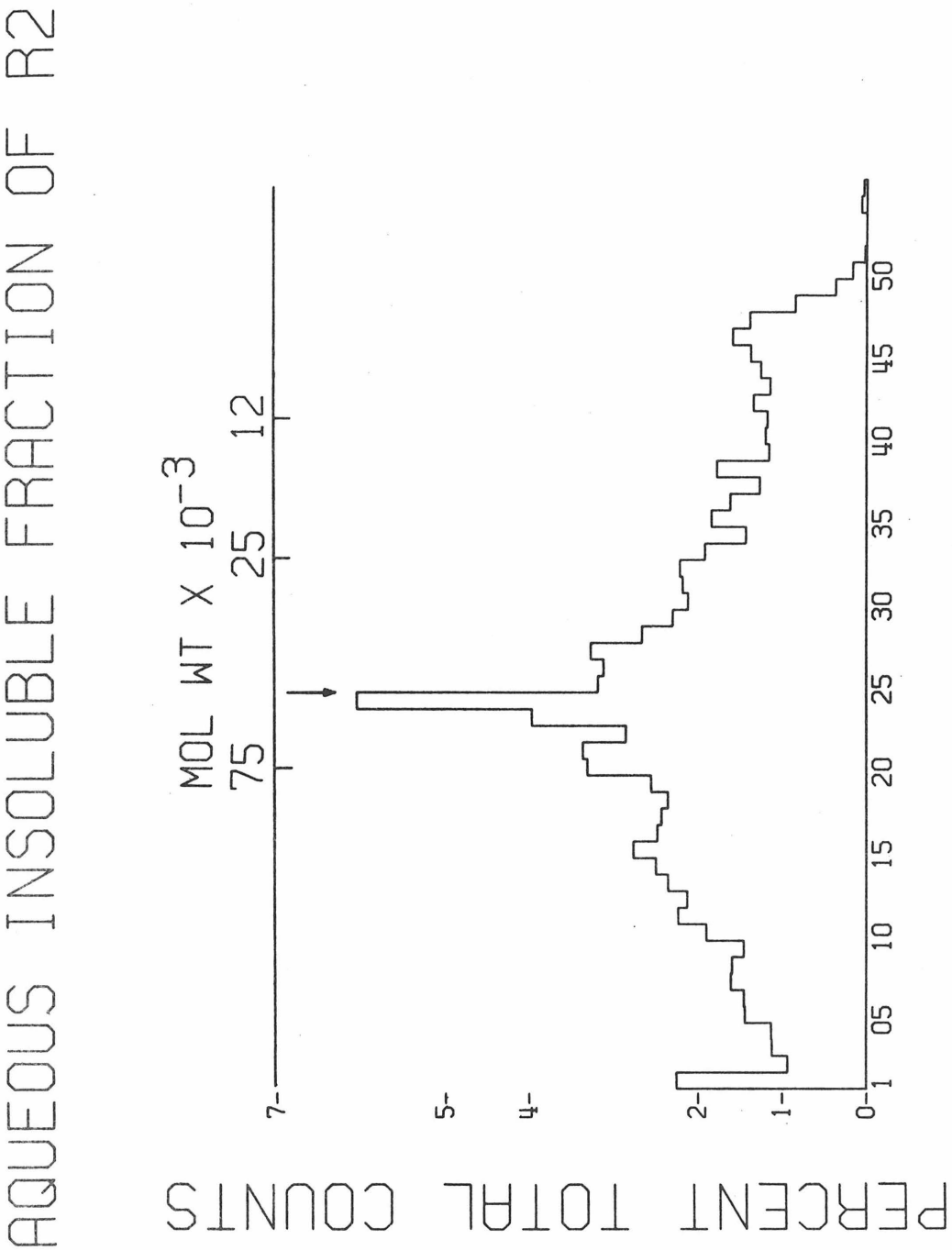

% COUNTS AT 50K
20
15
10
5
0
0
5
10
15
20
25
30
% AQ. SOL. PROTEIN

Figure 4. Gel pattern from the aqueous insoluble fraction of R2. This is a control pattern of ^{14}C-labeled peptides. Normal [K^+] was present in both the control and experimental phases of the experiment. Protein molecular weight calibration was determined by average migration relative to the bromphenol blue tracking dye.
Total ^{14}C counts in gel: 2354 cpm.


AQUEOUS INSOLUBLE FRACTION OF R2
MOL WT X 10^{-3}
75
25
12
PERCENT TOTAL COUNTS
7-
5-
4-
2-
1-
0-
1
05
10
15
20
25
30
35
40
45
50
SLICE NUMBER

2. The effect of high [K^+] media

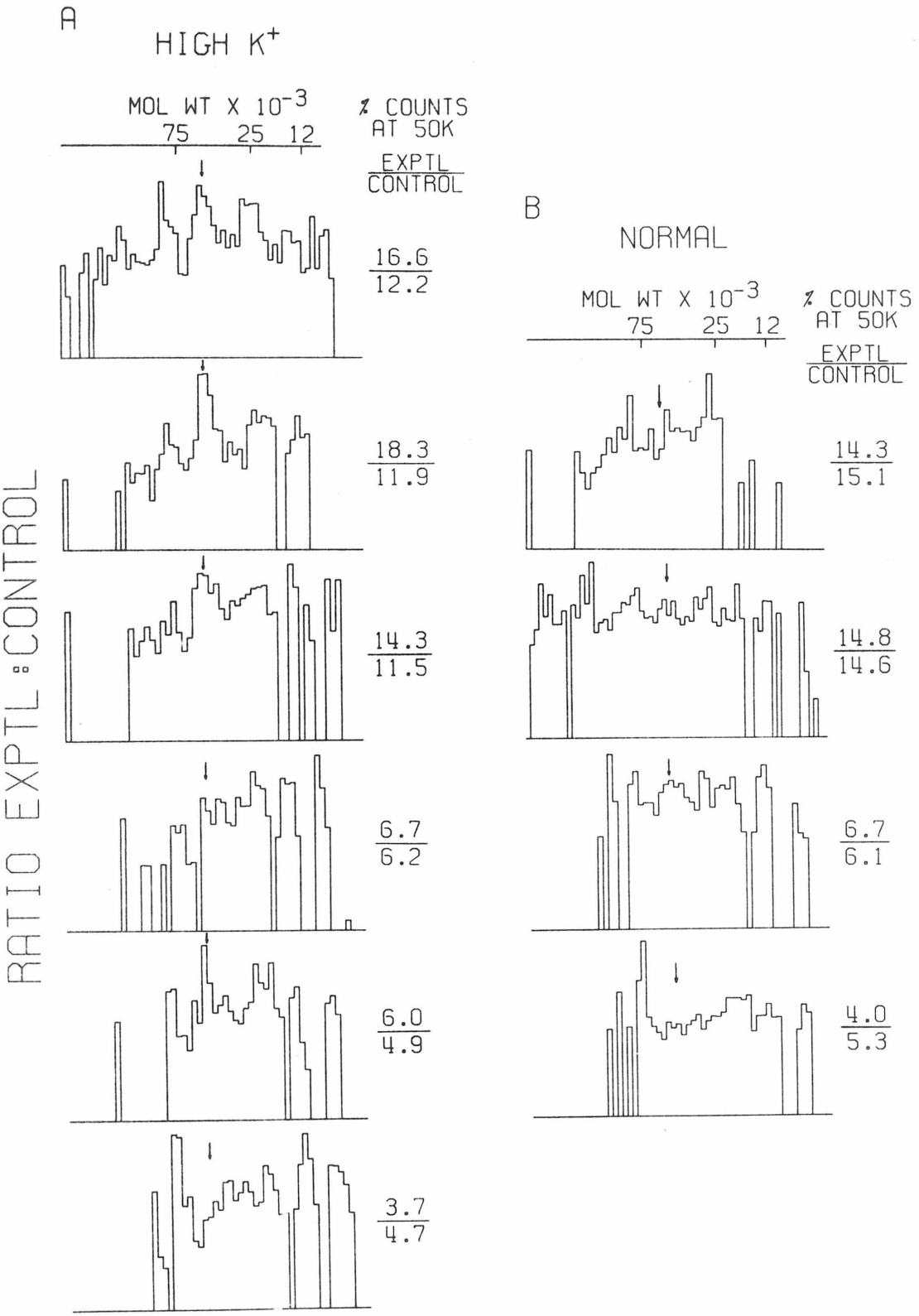
Aqueous soluble fraction. High [K^+] media caused a relative increase in incorporation into the 50K peak of the aqueous soluble fraction of the giant cell. This effect varied both qualitatively and quantitatively as the aqueous soluble incorporation pattern varied. Figure 5A shows patterns of the ratio of experimental to control counts obtained in high [K^+] experiments. The patterns are placed in the order of decreasing percent of total counts found in the 50K peak in the control pattern. Figure 5B shows ratio patterns obtained when the second incorporation was in normal media.

Statistically significant differences between the patterns obtained with high [K^+] incubations and those with normal incubations can be demonstrated. The peak size on the ratio bar graph for the 50K peak in the giant cell averages 1.1 $\pm$ .1 with normal incubations (N = 4) and 1.4 $\pm$ .2 with high potassium (N = 6) (significantly different: $p = .019$ on a Mann-Whitney U test; $p < .025$ on Student's t test).

These patterns also consistently show a peak at around 85K, whether high [K^+] was present or not.

Aqueous insoluble fraction. High [K^+] media may also cause a relative increase in incorporation into the 50K peak in the aqueous insoluble fraction of the giant cell. The pellets of giant cells from three normal [K^+]

Figure 5. Ratio of ^{3}H (experimental) counts to ^{14}C (control) counts for the aqueous soluble fraction of the giant cell. A. Experimental media contained either 90 or 110 mM $[K^{+}]$. B. Experimental media contained the control level of K^{+}, 10 mM. In both A and B the patterns are arranged in the order of decreasing incorporation into peptides at 50K in the control (^{14}C) pattern. The amount of incorporation into peptides at 50K, expressed as a percentage of the total aqueous soluble protein counts appearing in two slices located at 50K, is indicated for both experimental and control patterns. The arrow with each pattern indicates the position of 50K. These patterns are for all experiments in which the control pattern averaged more than 7 cpm/slice above background (15 cpm). Where a slice had fewer than 5 cpm above background the ratio was not computed and has been set equal to zero in these patterns.


A
HIGH K+
MOL WT X 10-3
75 25 12
% COUNTS AT 50K
EXPTL / CONTROL
16.6 / 12.2
18.3 / 11.9
14.3 / 11.5
6.7 / 6.2
6.0 / 4.9
3.7 / 4.7
RATIO EXPTL : CONTROL
B
NORMAL
MOL WT X 10-3
75 25 12
% COUNTS AT 50K
EXPTL / CONTROL
14.3 / 15.1
14.8 / 14.6
6.7 / 6.1
4.0 / 5.3

experiments were run on gels. A peak at 50K was obtained in the ratio patterns in two of the three high $[K^+]$ experiments. The normal $[K^+]$ experiments and the third high $[K^+]$ experiment did not show a peak at 50K.[1] Ratio patterns of a normal $[K^+]$ experiment and of a high $[K^+]$ experiment that showed the peak are shown in Figure 6. The peaks that appeared at about 85K in both of the patterns shown in Figure 6 were present in all six pellet samples.

Total counts incorporated. High $[K^+]$ caused a reduction in the total number of counts incorporated into the cell during the experimental incubation period. For the aqueous soluble fraction the ratio of total experimental counts on a gel to total control counts on a gel decreased in high potassium experiments to 65% of the ratio found in experiments with normal potassium throughout ($p < .03$ on both Walsh matched pairs signed ranks test and on t test for paired variates, N = 6 pairs[2]). For the

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[1]Some of the details of the "failure" that differed from the two "successes" are the following: (1) the high $[K^+]$ incorporation period was only 3/4 hour long, and (2) the sample was stored at 10°C for approximately 10 months before being run. All three high $[K^+]$ pellets were from R2 cells whose 50K "peak" in the aqueous soluble fraction had fewer than 8% of the total aqueous soluble protein counts.

[2]To convert data to an interval scale (necessary so that experiments with different experimental incubation periods or label specific activity could be compared) the test was done on the logarithms of total counts ratios. Matched cells were from ganglia incubated for the same

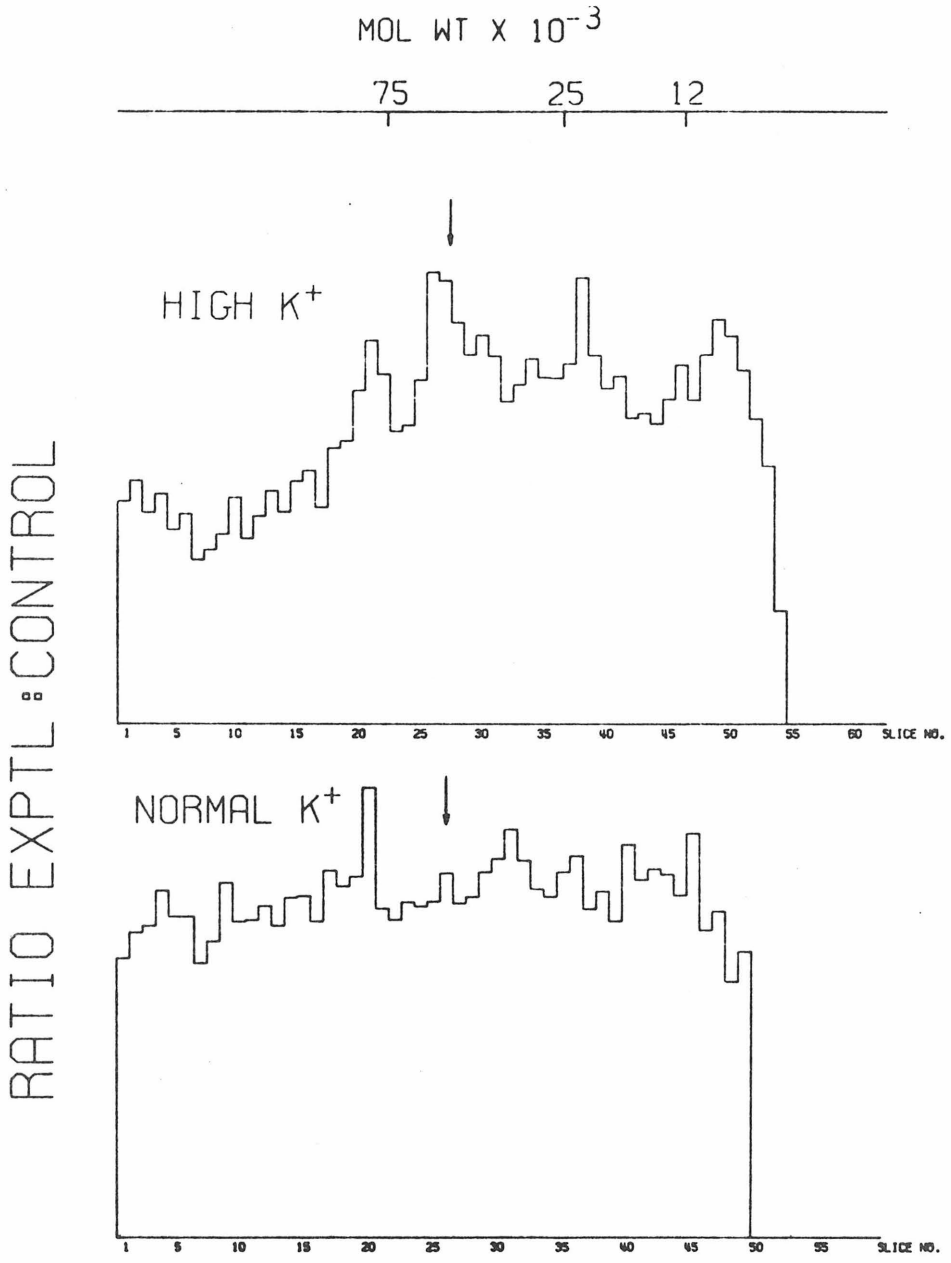

Figure 6. Ratio of ^3H (experimental) counts to ^{14}C (control) counts for the aqueous insoluble fraction of the giant cell. High K^+: Experimental media contained 90 mM [K^+]. Normal K^+: Experimental media contained the control level of [K^+], 10 mM.

aqueous insoluble fraction a similar decrease (to 68% of normal) was found ($p < .03$ on both t test for paired variates and Walsh matched pairs signed ranks test, $N = 6$ pairs. The Walsh test is described in Siegel, 1956).

DISCUSSION

Interpretation of the results presented in this chapter is complicated by the variability of the incorporation pattern of the aqueous soluble fraction. This variability may be due to the following factors: (1) intrinsic variation of the peptides synthesized by the giant cell, (2) variation in the amount of radioactivity in adhering glial and other contaminating cells, and (3) variability in the homogenization and solubilization conditions. The first possibility is unattractive since, if true, it would mean that reproducibility from animal to animal is not attainable. The first possibility will not be further considered while it seems likely that the variability could be due to artifacts of techniques, as suggested by possibilities two and three.

The amount of radioactivity in adhering cells may indeed vary. In the next chapter, in which measurements on autoradiographed sections of two dissected giant cells are

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length of time in label of the same specific activity on the same day.

reported, contaminating cells accounted for up to 11% and 34%, respectively, of the total formalin-fixed radioactivity in the dissected "cell." Variability of this magnitude could account for the variation observed at 50K; however, to do so, more than 50% of the glial incorporation would have to be at 50K.[1] A feasible way of checking this possibility would be to run gels on glial and neuronal tissue dissected from sections of giant cells cut on a cryostat. Kato and Lowry (1973) have recently reported on the regional localization of several soluble and insoluble enzymes within nerve cell sections micro-dissected in this manner.

Variability in the homogenization and solubilization conditions is considered the most likely explanation. A slight variation in the homogenizing conditions due to different amounts of ions and proteins contributed by the homogenized tissue could conceivably change the solubility of the proteins containing 50K peptides. It should be noted that in the whole ganglion experiments aqueous soluble protein concentration was much greater (as judged

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[1]This figure is arrived at as follows: Percent of total counts of 50K varied from about 5% to 15%, a range of 10%. The range of the two autoradiographed cells was about 20%. To account for the variability at 50K, 50% of the glial incorporation would have to be at 50K, and the decrease at 50K would have to be the only change (If glial contribution at other molecular weights also decreased, an even higher percentage would have to be at 50K).

by amount of staining on gels with Coomassie Blue), and ions in the ganglion raised the ionic strength of the homogenizing buffer. It may be of significance that the percentage of ^{14}C-labeled protein which was aqueous soluble averaged only 12% in these giant cell experiments, but was 20% in comparable internal control experiments on hemiganglia. Conditions equivalent to those used in ganglion experiments should be tested, perhaps by homogenizing single cells in a buffer containing a homogenate of an unlabeled ganglion. Such a procedure would have the further benefit of providing a staining pattern for better correlating peaks in the labeled cell to the gangion pattern.

The results indicate that high $[K^+]$ caused a relative increase in incorporation at 50K in the aqueous soluble fraction of the giant cell. This is most clearly seen by looking only at those cases in which the control patterns contained more than 11% of their label in the 50K peak (see Figure 5). Three experiments with high $[K^+]$ during the experimental period showed sizeable increases at 50K, whereas two experiments with normal $[K^+]$ during the experimental period did not. The percent of total counts at 50K increased in the three high $[K^+]$ experiments an average of 4.5%, while the two normal $[K^+]$ experiments showed a mean decrease of .3%. ($4.5 \pm 1.6\%$, $-.3 \pm .7\%$; $p < .002$, Student's t test). The relative increase at 50K caused by high $[K^+]$ is in agreement with observations made on the

whole ganglion. High $[K^+]$ caused a decrease in total amount of incorporation into the giant cell, also in agreement with the decrease in total incorporation seen in the whole ganglion.

The variability in the absolute size of the 50K peak in the control patterns may make one suspicious that $[K^+]$ is merely manipulating whatever factor is responsible for the variability. One finds, however, that high $[K^+]$ did not change the percent of counts found at 50K in the ^{14}C control pattern. Due to the internal control method used in the experiment, such an objection must suppose that $[K^+]$ has an effect only on recently synthesized peptides. A test on this possibility might be to use two consecutive normal incubation periods with different labels, followed by a short exposure to high $[K^+]$. This experiment has not been done.

The incorporation pattern of the aqueous insoluble proteins of the giant cell showed a peak at 50K, and in two out of three cases high $[K^+]$ caused a sizeable increase in labeling at 50K. This is not enough data to achieve statistical significance. It is not known whether the peptides that did increase were identical to the 50K peptides that increase in the aqueous soluble fraction. Tubulin subunits, which are near this molecular weight, are one example of a protein which partitions into both aqueous soluble and aqueous insoluble fractions (Barondes & Feit,

1970). The aqueous insoluble fraction of the whole ganglion showed a much less prominent peak in the incorporation patterns at 50K (Figure 6, Chapter II) and did not show a relative increase at 50K due to high $[K^+]$ (N = 5). A possible explanation for the difference between the ganglion and the giant cell lies in the different partition of proteins into aqueous soluble and aqueous insoluble fractions, discussed in the beginning of this section.

Both the aqueous soluble and the aqueous insoluble fraction of the giant cell showed peaks at 85K in the ratio patterns regardless of whether the second incubation was in high $[K^+]$ or normal medium. This is reminiscent of the 85K peak which appeared in the aqueous soluble fraction in internal control experiments on the ganglion (Chapter II). The cause of this peak is unknown. It may be due to a change in synthetic capacities of the tissue due to being dissected from the animal, or it may represent the result of processing or exporting 85K peptides.

The most important result of this chapter is that high$[K^+]$ media caused a relative increase in incorporation at 50K at the level of the single dissected neuron. Unless this increase is taking place in adhering glial cells, this result indicates that the similar change in incorporation at 50K observed in the aqueous soluble fraction of the whole ganglion reflected changes taking place in individual

neurons of the ganglion. The possibility that changes in the glial cells adhering to R2 could be responsible for this phenomenon is examined in the next chapter.

SUMMARY

The effect of high [K^+] media on the pattern of incorporation of leucine into protein of the giant cell (R2) of the parieto-visceral ganglion of Aplysia californica was examined. Ganglia were first labeled with ^{14}C-leucine in normal media and subsequently labeled with ^{3}H-leucine in either high [K^+] or normal media. The ^{14}C pattern acted as an internal control for individual cell patterns. Experiments with normal media in the second incubation controlled for possible changes in pattern which were the result of the timing of the incubations. The ^{14}C control patterns of the aqueous soluble fraction of R2 were very variable; the percent of aqueous soluble protein counts appearing at 50K in this fraction was correlated with the percent of total protein counts which were aqueous soluble. High [K^+] caused a significant relative increase in incorporation at 50K, particularly in those cells showing a large amount of incorporation (> 11% of aqueous soluble protein counts) at 50K in the ^{14}C control pattern. High [K^+] also decreased the total incorporation into the R2 to about 65% of normal. These results were interpreted as providing support for the notion that similar changes in

the whole ganglion reflect changes taking place in single neurons.

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SUPPLEMENT TO CHAPTERS II AND III

ADDITIONAL ANALYSIS OF DATA

In addition to the changes in ratio patterns caused by high $[K^+]$ which have already been discussed, a number of other changes occurred which may be of some interest. In both R2 and the PVG high $[K^+]$ caused a greater decrease in incorporation at higher molecular weights than at lower molecular weights. This is most clearly seen in the aqueous insoluble fraction of the PVG, shown in Figure 5, Chapter II. Such an effect is also present, though to a smaller extent, in dissected R2s and in the aqueous soluble fraction of the PVG, and to an even smaller extent, in the aqueous soluble fraction of R2.

METHOD OF ANALYSIS

To analyze this effect statistically, least squares lines were fitted to the ratio patterns obtained from these experiments. Ordinarily, in doing a least squares fit, one would take into account the variance for each point. As shown in Figure 2, Chapter II, the variance of each point in the ratio pattern was different, due to differences in counting variance along the gel. Taking these different variances into account would have unduly stressed regions of the gel having more counts--regions such as 50K in the aqueous soluble pattern, which is known to be exceptional

in the ratio observed there. Variances were therefore assumed to be equal along the entire ratio pattern for purposes of calculating a least squares line.

It was also necessary to normalize the slopes obtained. Thus, we would want a sample in which the ratio decreased from 7 at one end to 3.5 at the other to have the same slope as a sample in which the ratio decreased from 6 at one end to 3 at the other. Such differences between samples are likely to be due to trivial causes, such as differences in size of two ganglia used in parallel control experiments. Slopes were normalized by normalizing the points from which they were plotted, using the following equation:

$$R_{jnormalized} = \frac{\frac{1}{2}(N-1)N}{\sum_{1}^{N} R_i} R_j$$

where N is the number of slices for which ratios were calculated (all slices having more than 5 cpm above background), and R_j are the ratios which are being normalized. For samples in which ratios were calculated for all slices from the top of the gel to the fastest moving proteins, the slope of a least squares fit to these normalized ratios gives the fractional increase of the ratio at the fast moving end of the fitted line to the ratio at the midpoint of the line. Thus, a line that increased 100% between the midpoint and the end would have a slope of 1.00, a level

line would have a slope of 0.0, and a 50% decrease between the midpoint and the end would give a slope of -0.50. The least squares fit was made according to standard formulae (Bliss, 1967).

Ratios were calculated for all slices from the top of the gel to where the fastest proteins migrated for almost all PVG samples. The exceptions (e.g. Figure 10 in Chapter II), left out only a few slices at most. R2 samples, on the other hand, generally had several slices in the middle of the gel for which ratios were not calculated (see Figure 5, Chapter III). The method of calculation used here would underestimate the slope, but would give the right sign for samples in which ratios in the middle have not been calculated.

RESULTS

The results of these calculations are shown in Table I. The results for parallel control experiments showed a large, significant positive slope when high $[K^+]$ (90-110 mM) was used in the experimental media. The effect was largest in the aqueous insoluble fraction of the PVG. In this fraction, 50 mM $[K^+]$ also caused a small effect of this sort.

The positive slope caused by high $[K^+]$ was smaller in the aqueous soluble fraction than in the aqueous insoluble fraction. There was too much scatter in the

TABLE I. SLOPE OF LEAST SQUARES FIT TO RATIO BAR GRAPHS

Type of incubation	$[K^+]$ in exptl. media (mM)	Type of sample	Slope ± S.D.	N	p (on t test)
Sim. inc.	10	PVG-sol	-.023 ± .017	3	n.s.
Sim. inc.	90	PVG-sol	-.067	1	
Sim. inc.	10	PVG-insol	-.033,-.027	2	
Parallel	10	PVG-sol	-.037 ± .115	9	
Parallel	90-110	PVG-sol	.328 ± .084	10	p<.01
Parallel	50	PVG-sol	.119 ± .181	3	n.s.
Parallel	Reduced Na^+	PVG-sol	-.032,.066, .412*	3	
Parallel	10	PVG-insol	-.049,-.011	2	
Parallel	90	PVG-insol	.592 ± .175	5	p<.01
Parallel	50	PVG-insol	.133,.101	2	p<.03
Internal	10	PVG-sol	-.131 ± .095	5	
Internal	90-110	PVG-sol	.015 ± .206	9	n.s.
Internal	10	PVG-insol	-.062	1	
Internal	110	PVG-insol	.378	1	
Internal	10	R2-sol	-.015 ± .066	4	
Internal	90	R2-sol	.118 ± .071	6	p<.01
Internal	10	R2-insol	.115 ± .069	3	
Internal	90	R2-insol	.338 ± .111	3	p<.02

*The value of .412 is somewhat misleading. The pattern for this sample was near zero slope up until a very large peak at the low molecular weight end, not seen in the other two samples of this type. The large peak at one end produced a least squares fit with a large slope.

slopes obtained in "reduced Na^+" experiments to say, from this data at least, that the effect was not caused by low $[Na^+]$. More detailed inspection of the ratio patterns, as indicated in the footnote to Table I, showed that reduced $[Na^+]$ probably does not produce a significant change in slope.[1]

Internal control experiments showed effects in the same direction as those obtained in parallel control experiments, but surprisingly these results do not show statistical significance. The difficulty may well be due to the fact that a straight line just is not a very good fit to these patterns, three of which are shown in Figures 8 and 9, Chapter II. The decrease in ratio at the low molecular weight end gets played against a great deal of variability at the high molecular weight end, where the counts are low. The results are slopes that vary a great deal.

This problem did not occur in the internal control patterns of R2. These showed significant positive slopes in high $[K^+]$ experiments for both the aqueous soluble and aqueous insoluble fractions.

Slopes have also been computed for ratio patterns

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[1]Alternatively, one may eliminate the one aberrant slope as an outlier, according to the Dixon gap test (Bliss, 1967). In that case, comparison of "reduced Na^+" with "high K^+" gives $p < .01$ (t-test). No significant difference from normal media experiments is found.

obtained from pleuro—visceral connective nerves. The patterns do not, however, lend themselves well to a linear regression since they have two broad peaks of very variable magnitude at the high molecular weight end (see Figure 10, Chapter II). A slope of -.250 $\pm$ .680 was obtained (N = 5).

DISCUSSION

High $[K^+]$ has been shown to have the general effect of lowering relative incorporation into higher molecular weight peptides. An effect of $[K^+]$ is seen as low as 50 mM $[K^+]$ but is much more pronounced at 90 mM.

One surprising aspect of the data is that slopes for simultaneous incorporation experiments were always slightly negative. This could be due to differences in the metabolism of the label on ^3H- and ^{14}C-labeled leucine, or due to compounds other than leucine being in the labeled precursors (see Appendix B). Alternatively, a slight error in the estimation of the background would have the effect of slightly raising or decreasing the ratio of counts, particularly where the counts were not very much above background. For example, the counts are generally lower at the high molecular weight end; the top slice may have only 10-20 cpm of ^{14}C above background. An error of only 1 cpm in the background could increase or decrease the ratio by 5-10% for counts this low. Background counts were generally determined by averaging the counts in the several

slices in a gel (or gels, if several were run) which were beyond the fastest running proteins. Depending on the counter, type of vial, gain setting, and window setting used, backgrounds were between 10 and 20 cpm for both ^{14}C and ^{3}H.

To test whether an error in estimating the background could really be responsible for the slightly negative slopes, the ^{14}C background used for one such sample (Figure 2, Chapter II) was varied and the slopes were calculated. As the background was varied from 12.0 to 15.0 cpm. the slope changed from +.010 to -.018, respectively. A background of 15.0 had actually been used for Figure 2, Chapter II. Therefore, it does seem possible that an error in estimating the background could produce these unexpected negative slopes. It is worth noting, however, that errors of this type would be too small to produce any of the large effects on slopes caused by high $[K^+]$ incubation.

An error in background estimation would also have a slight effect on the 50K peak height in a ratio bar graph. In the example used in the previous paragraph, as the ^{14}C background was varied from 12.0 to 15.0, the 50K peak size changed from 1.014 to .995, respectively. Again, errors of this sort would be too small to produce the much larger effects caused by high $[K^+]$ incubation.

It is hard to imagine how the relative decrease in

incorporation into proteins of higher molecular weights in response to high [K^+] might have adaptive value for the tissue. One could imagine that a selective change of relative synthesis of a few proteins, as actually occurred at 50K might be a functional response. One might also imagine that decreasing total synthesis, as actually occurred, might be the way a cell functionally adapts to an increased demand for energy by other processes, such as active transport. It is difficult to imagine why lower molecular weight peptides, per se, would be more useful to have than higher molecular weight peptides.

None of these experiments gives an indication of the mechanism of this general effect of high [K^+] incubation on incorporation into higher molecular weight peptides. It is tempting to speculate about the mechanism involved. One possible explanation would be that high [K^+] incubation caused nascent peptides to fall off ribosomes prematurely.

CHAPTER IV

AUTORADIOGRAPHIC STUDIES OF THE INCORPORATION OF ^{3}H-LEUCINE INTO TWO IDENTIFIED NEURONS OF *APLYSIA CALIFORNICA*

Several studies have investigated the incorporation of leucine into neurons dissected from ganglia incubated in media containing radioactive leucine (Wilson, 1971; Schwartz, et al., 1971; Gainer, 1972; Wilson & Berry, 1972, and this thesis). In interpreting the data presented in this thesis and these other references a question of crucial importance was, how much of the incorporation into the dissected neuron was actually in the neuron and how much was in the glial cells which always adhere to it. One study which bears on this question is that of Strumwasser (1967, also Strumwasser & Bahr, 1966) which showed autoradiographs of the right bag cell cluster and several right upper quadrant cells of the parieto- visceral ganglion (PVG) of *Aplysia*. In these cases few, if any, of the grains were shown between the cells, where the glial cells would be located. In another study, Schwartz, et al. (1971) dissected cells R15 and R2 (nomenclature of Frazier, et al., 1967) which had been incubated in media containing labeled leucine and then isolated the nuclei of these cells. The 25% of total counts in the cells associated with the nuclei was presumed to be the lower limit of

truly neuronal protein synthesis in the dissected cells. Berry (1972) similarly separated R2 nucleus, cytoplasm, and "remainder" (containing the cell membrane and adhering glial cells and cytoplasm). He found 15% of the counts in the nucleus, 10% in the cytoplasm, and 75% in the "remainder."[1] These studies indicated a need for a closer look at the relative amounts of label in the neuronal and glial regions of dissected cells. In this chapter, I report on autoradiographic studies of incorporation of radioactive leucine into identified cells and their adjacent glial cells. These studies have been carried out both in sections of whole ganglia and in sections of dissected cells. These results show that the preponderance of label found in labeled dissected neurons is neuronal in origin.

MATERIALS AND METHODS

PVGs were dissected from Aplysia as described previously (Chapter II). Following dissection PVGs went through two 90 minute rinses in normal blood salts, followed by a 240 minute incubation in normal incubation medium containing ^{3}H-leucine (media described in Table I,

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[1]These percentages were calculated by me from the number of counts found in gel patterns of these fractions, which Berry has communicated to me (1972). The calculation does not include the top six slices of the gels, which contain free leucine.

Chapter II). At the end of the incubation period, PVGs were rinsed for 10 minutes in normal blood salts media. To study the giant cell (R2) in intact ganglia, ganglia were dipped briefly (5-10 sec) in Tissue Tek Embedding Medium (Ames Company, Elkhart, Indiana) at room temperature and frozen in a large drop of Tissue Tek placed a few seconds before on a stage in an American Optical cryostat having an ambient temperature of -23° C. Alternatively, identifiable neuronal somata were dissected fron the ganglion (Wilson, 1971), sucked up whole into a pipette, and then gently pipetted onto the cryostat stage on which a drop of Tissue Tek had just been placed. Tissue was sectioned at 15 μ, picked up on warm slides, and dried on a warming table at 28° C. Sections were fixed for 15 minutes in buffered 10% formalin (Humanson, p. 14, 1962) and then washed in tap water 12 to 18 hours. After drying for at least a few hours, sections were either stained directly (see below) or autoradiographed by dipping slides in NTB2 (1:1 dilution in water) at 40° C (NTB2 is Nuclear Track Bulk emulsion, Eastman Kodak, Rochester, N. Y.). Slides were allowed to dry vertically for one or two hours and then stored for one to seven days at 2° C, with dessicant. Slides were developed in Kodak D-19 Developer at 17-19° C for three minutes, rinsed five minutes, fixed five minutes in Kodak Fixer, and rinsed in tap water 15-30 minutes before being stained and mounted.

Staining was done with 0.5% cresyl violet followed by differentiation in an alcohol series. A typical protocol, which was varied according to the age and strength of the staining solution was:

0.5% cresyl violet	5 min.
rinse in water	5 min.
70% ethanol	5 min
70% ethanol	5 min
95% ethanol	2.5 min.
100% ethanol	2.5 min.
100% ethanol	2.5 min.
Xylenes	5 min.-several hours
Xylenes	5 min.

Mount in Permount (Fisher Scientific, Fairlawn, N. J.).

Grains on autoradiographs were counted under an oil immersion objective (100X) using an eyepiece net micrometer disc. The micrometer disc had boxes approximately 69 μ^2 in area at this magnification as measured with a stage micrometer. Background was counted for each section in a blank area of the slide adjacent to the section. The median average background observed was .8 grains /69 μ^2 (range .3 to 3.9).

RESULTS AND DISCUSSION

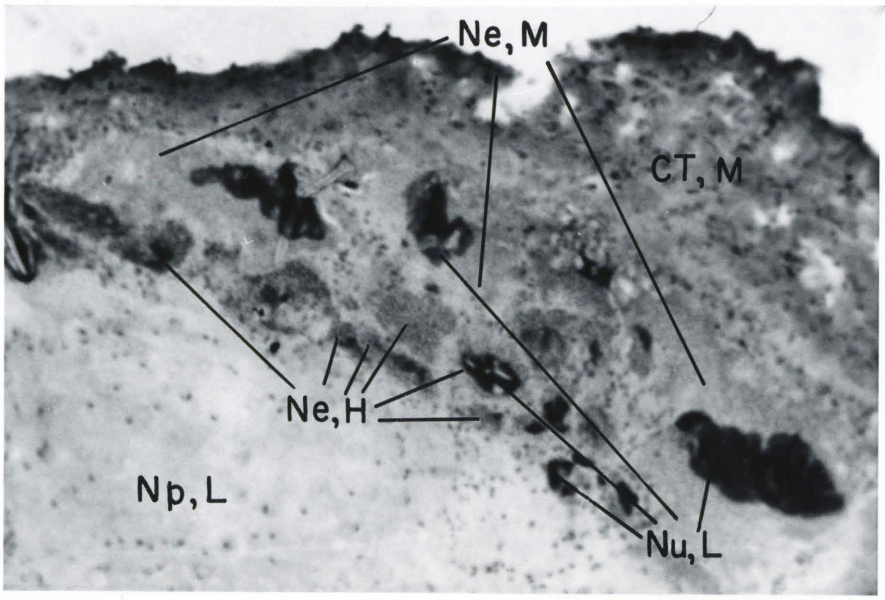
1. Whole ganglia

A casual observation of autoradiographs of sectioned ganglia confirmed the observations of Strumwasser (1967) that labeling in the glial cells was much lower than in neurons and that neurons within a section differed from one another in their grain densities (Figure 1). Figure 1 also demonstrates that variation between neurons was not caused by local variations in efficiency of autoradiography since the same relative differences were seen in serial sections. It is tempting to suppose that such differences among neurons reflect different functional levels of protein synthesis in different neurons; however, artifactual differences might arise due to unequal precursor availability or, perhaps, unequal availability of oxygen.

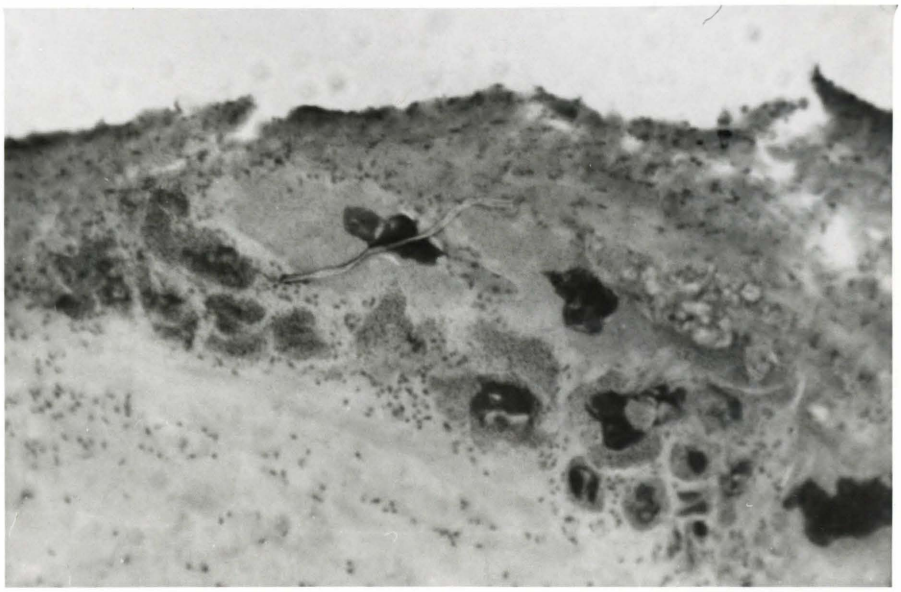
These autoradiographs show clearly that incorporation into neuronal cytoplasm is much greater than into neuronal nuclei. This is to be expected since the ribosomes, the protein synthesizing machinery of the cell, are located in the cytoplasm.

The giant cell, being the largest cell in the ganglion, could easily be identified in sections of whole ganglia (Figure 2). In several sections of each of three ganglia, grain densities over the giant cell cytoplasm and patches of nearby glial cells were counted.

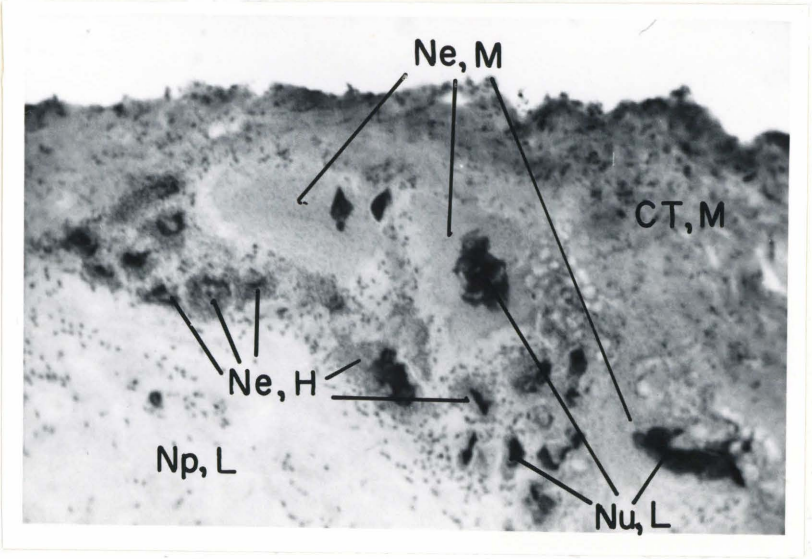
Figure 1. Autoradiographs of sections from a PVG. (a, b, c) Three serial sections of the lower right ventral region of the PVG. (d, e) Higher power magnifications of two regions on section c. Nu, nucleus; Ne, neuron; G, glial cell; Np, neuropil region; CT, connective tissue sheath. Relative densities of grains: H, heavy; M, medium; L, light. These are sections from PVG 180, sections 52-54 (see Table I).



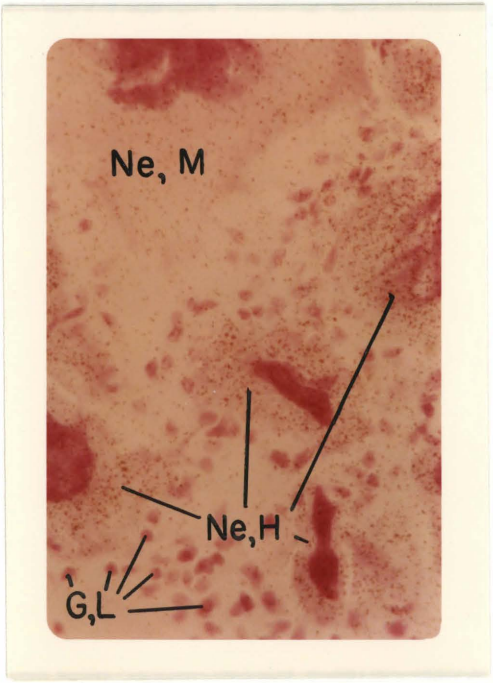
(a)



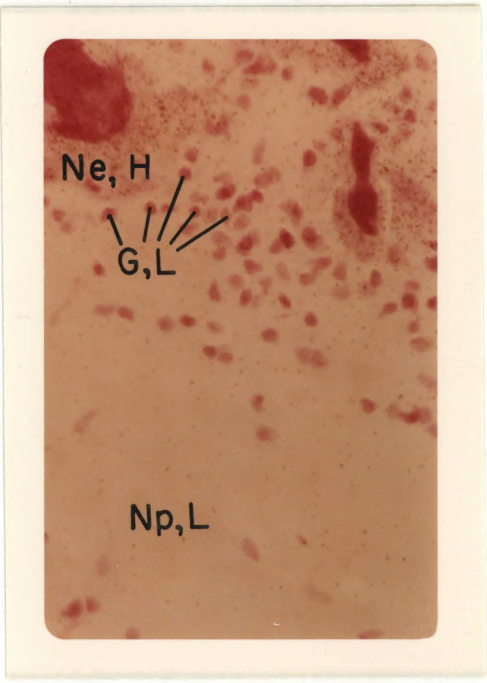
(b)



(c)



(d)



(e)

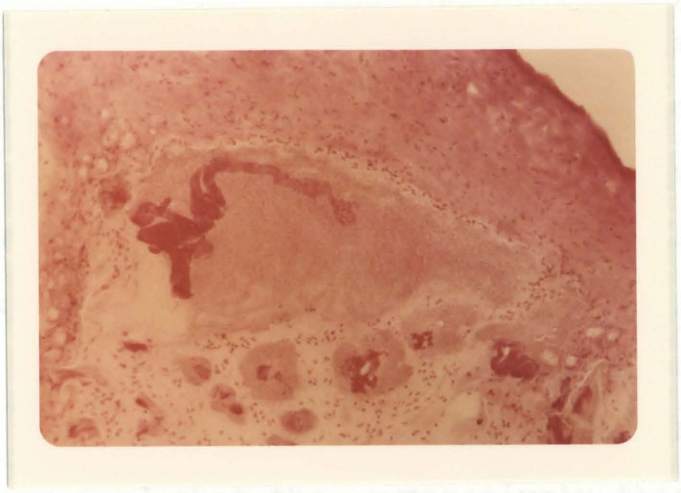


Figure 2. Autoradiograph of upper right dorsal region of the same section shown in Figure 1c. The large cell in the center is R2. Abbreviations as in Figure 1.

These patches of glial cells were located adjacent to the giant cell towards the center of the ganglion. These patches were always at least 500μ^2 in area and sometimes as much as 2400μ^2 could be counted. It was not always possible to tell where there was no neuronal cytoplasm by the appearance of the staining. As a rule, areas showing glial morphology (i.e. glial nuclei were present) and having several adjacent boxes of low grain density were counted as glia. This rule, of course, produces a bias against counting glia which may incorporate heavily. In fact, it appeared that the comparatively thin strip of glia at the outer margin of the giant cell incorporated more heavily than other glia; however, it is difficult to accurately distinguish what is neuronal and what is glial in this region (Rosenbluth (1963), comments on the difficulty of distinguishing neuronal from glial cytoplasm in Aplysia). Grains over the giant cell cytoplasm were generally counted in a 3 box x 3 box region near the nucleus of the cell. In two of the ganglia, the grain density appeared to be fairly uniform throughout the cell. In the third ganglion, R2 showed a distinct perinuclear region in both the staining and grain density. In this case the perinuclear region had about twice the grain density of the ectoplasm. For purposes of calculations, the grain density of the cytoplasm was taken as the mean of these two densities. The median ratio of grain densities minus

background of giant cell cytoplasm to nearby glial cells was 14 :1 (see Table I).

The density of grains over R2 appeared to be fairly typical of most neurons in ganglion sections. The relative volumes occupied by neuronal cell bodies and the glia between them was not measured in detail; however, it appears unlikely that the latter occupied more than twice the volume of the former. Given the relative grain densities observed in R2 cytoplasm and adjacent glial cells, this would mean that total incorporation into glial cells is less than 20% of that into neurons in the ganglion.

Another observation which may be of some interest is that little, if any, incorporation occurred in the neuropil region of the ganglion (see Figure 1). The grain density in the neuropil was equal to or less than the background density as often as it was greater than the background.[1] This indicates that glial cells in the neuropil, like glial cells closer to neurons, do not incorporate very much label. This may be due to either lack of precursor availability or inherent low levels of synthesis. This result would also indicate that the neurons in the

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[1] In one PVG there were a few "hot spots" in the neuropil having densities as high as about half that seen in R2 cytoplasm. These spots were very infrequent and not seen in other ganglia.

TABLE Ia.

R2 CYTOPLASM : ADJACENT PATCHES OF GLIA

(a summary of data in TABLE Ib.)

Sample	Ratio of grain densities[1], R2 cytoplasm:adjacent patches of glia		
	individual sections[2]	median	overall median
PVG 180	9.9, 9.2, 7.9; 22.8, 9.9, 7.2; 12.5	9.9	
PVG 181	31.4, 16.1; 11.5, 10.3, 13.7; ∞	14.9	13.8
PVG 182	16.7; 240.2, 25.1; 13.9; ∞	25.1	

[1]Background subtracted.

[2]Sections separated by a comma are serial sections on single slides; semicolons separate different slides.

TABLE Ib. GRAIN DENSITIES

OF R2 CYTOPLASM AND ADJACENT GLIA

PVG #	Section #	Grain density (grains/69 μ^2) A R2 cyt.	B glia nr. R2	C bkgd.	A-C / B-C	B-C / A-C
	180,52	21.7	2.9	.8	9.9	.10
	180,53	27.1	4.0	1.2	9.2	.11
	180,54	31.9	5.1	1.2	7.9	.13
180	180,45*	21.4	1.7	.8	22.8	.044
	180,46*	12.4	1.7	.5	9.9	.10
	180,47*	21.7	3.8	.9	7.2	.14
	180,61	16.7	1.8	.5	12.5	.080
	181,57	38.2	1.7	.5	31.4	.032
	181,58	65.3	4.8	.8	16.1	.062
181	181,51	75.5	7.1	.6	11.5	.087
	181,53	59.0	6.1	.4	10.3	.097
	181,53	66.7	5.7	.9	13.7	.073
	181,63	66.8	.9	.9	∞	0
	182,46	39.6,14.4	1.9	.3	16.7	.060
	182,42*	70.9,26.8	1.0	.8	240.2	.004
182	182,43*	90.5,41.9	3.6	1.0	25.1	.040
	182,40	57.2,25.4	3.8	.9	13.9	.072
	182,52	25.3	.6	.8	∞	0
median					13.8	.072

*Exposed 2 days. All other slides were exposed for 1 day.

Sections which were on one slide are grouped together. For explanation of two densities given for PVG 182, see text.

ganglion do not transport a significant amount of labeled proteins into or through the neuropil within the four hour incubation period of this experiment.

2. Dissected cells

Dissected cells were analyzed by measuring the areas and grain densities of the neuron and surrounding cells (mostly glia) on sections near the middle of the neuron. Only sections retaining reasonably good morphology were counted. The border between the neuron and surrounding cells was often difficult to define precisely. This was particularly true for about half the circumference of R2 cells, where a thin layer of glial cells was found with relatively dense grains (for glial cells) over them. Presumably these glial cells correspond to the glial cells on the outer margin of R2, which had relatively higher densities on whole ganglion autoradiographs. When in doubt, the region in question was assigned to the surrounding cells, and therefore the results probably underestimated the neuronal contribution.

An estimate of the total relative contributions of the neuron and surrounding cells was made by assuming these sections to be central sections of a spherical neuron with a thin shell of surrounding cells.

According to this model

$$\frac{V_s}{V_n} = \left(1 + \frac{A_s}{A_n} \right)^{3/2} - 1$$

$$\left[\frac{\text{Grains}_s}{\text{Grains}_n}\right]_{\text{sphere}} = \frac{A_n}{A_s} \frac{V_s}{V_n} \left[\frac{\text{Grains}_s}{\text{Grains}_n}\right]_{\text{section}}$$

Where the variables are defined as

V_s, total volume of shell of surrounding cells

V_n, total volume of neuron

A_s, total area of surrounding cells on a section[1]

A_n, total area of neuron on a section

$\left[\frac{\text{Grains}_s}{\text{Grains}_n}\right]_{\text{section}}$, ratio of total number of surrounding cell grains to total number of neuron grains observed on a section

$\left[\frac{\text{Grains}_s}{\text{Grains}_n}\right]_{\text{sphere}}$, ratio of total number of surrounding cell grains to total number of neuron grains calculated for model sphere.

This calculation probably overestimates the relative contribution of surrounding cells since

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[1]Relative areas were measured by projecting the section with a projection microscope, tracing on a piece of paper, cutting out the relevant regions, and weighing them.

cytoplasmic and nuclear grains are averaged together over the entire neuronal region of the section. Since the nuclear region has a lower grain density than the cytoplasm, this underestimates the total number of grains in the neuronal sphere. The actual ratio of total surrounding cell grains to total neuron grains is therefore probably somewhere between the calculated ratio and the ratio observed in individual sections. The factor which converts between these two, $\frac{A_n}{A_s}\ \frac{V_s}{V_n}$, averaged about 1.8.

Two dissected R2 cells and two dissected R15 cells were analyzed in this way. Data for these is shown in Table II. The two R2 cells had 11% and 34% of their radioactivity in the surrounding cells. R15 cells had 12% and 16% of their radioactivity in the surrounding cells. These cells were estimated by the model to be approximately 65% non-neuronal by volume. Figure 3 shows sections and schematic analyses of an R2 and R15 cell.

The results in this chapter confirm and extend the findings of Strumwasser (1967). Whole ganglion autoradiographs showed that glial cells adjacent to the giant cell incorporate little ^{3}H-leucine. The similarity of grain distribution around dissected neurons to the grain distribution around neurons in ganglion sections serves to validate the dissection technique used.

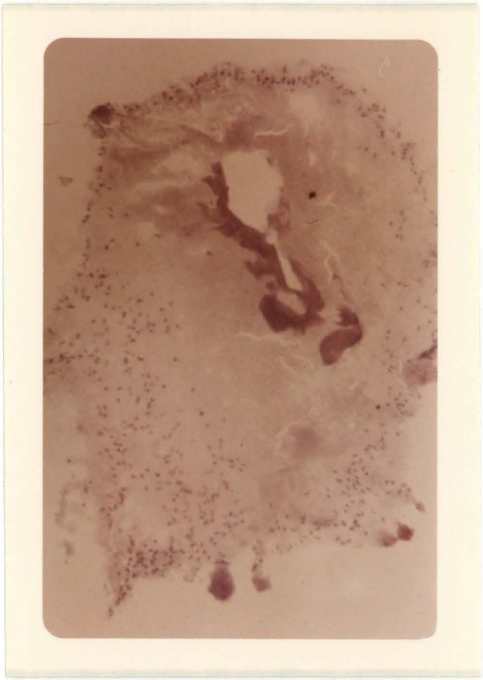
The experiments on dissected cells demonstrate that

TABLE II.

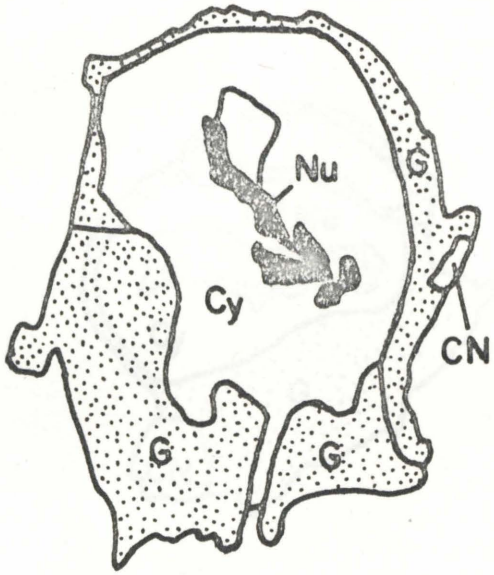
RELATIVE CONTRIBUTIONS OF NEURON AND SURROUNDING CELLS TO DISSECTED CELLS

sample			area	volume	total grains		
PVG	cell name	slide #	per cent surr. cells on section	calculated per cent surr. cells in model	per cent surr. cells on section	calculated per cent surr. cells in model	mean
187	R2	23-24	44	58	5	9	11
		25-26	52	66	8	14	
189	R2	26-27	46	60	19	30	34
		20	51	65	25	38	
187	R15	18	55	69	6	11	12
		16	57	71	3	13	
188	R15	12	59	73	10	19	16
		13	44	59	8	13	
	mean		51	65	10	18	

Figure 3. (a) Autoradiograph of a section of a dissected R2. (b) Schematic diagram of regions into which the section was divided for counting grains. (c) Higher power magnification of part of the section shown in (a). (d, e, f) Same as (a), (b), and (c) for a section of a dissected R15. Nu, nucleus; Cy, cytoplasm of neuron; G, glial cells; CN, contaminating neuron. These sections are R2, 187,23-24 and R15,187,16 (see Table II).



(a)



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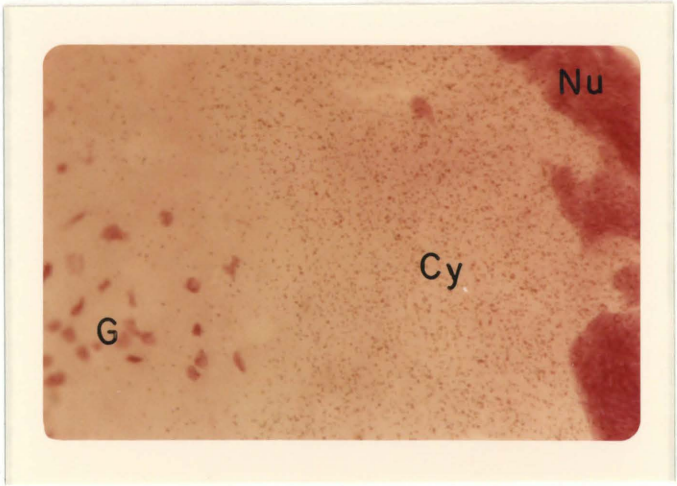

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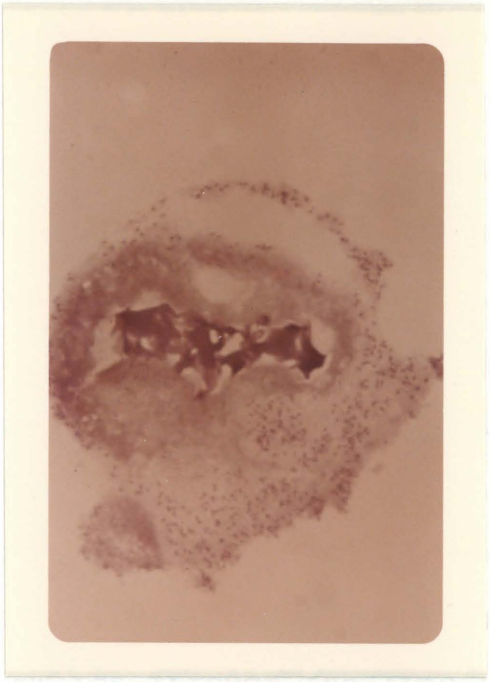
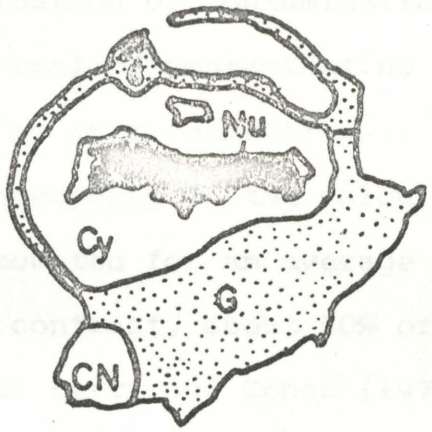
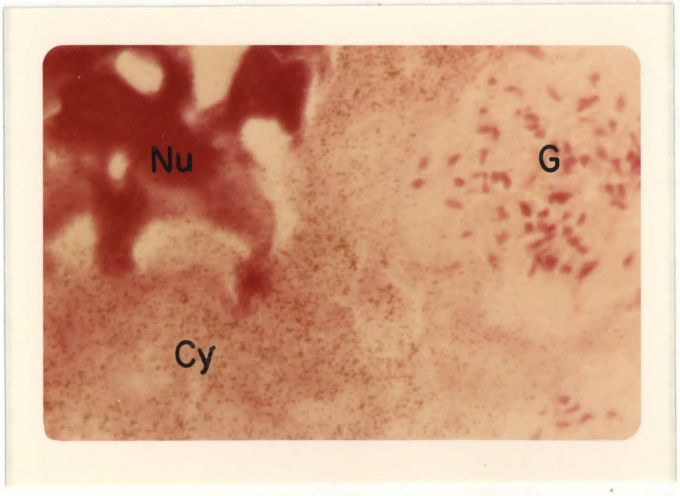


(d)



(e)



(f)

glial cells adhering to two identified cells in the PVG generally account for less than one-fifth of the leucine incorporated into them. In considering this figure, it would be well to keep in mind the fact that this analysis overestimated the relative contribution of contaminating cells in assigning grains to neuronal or contaminating cell regions and in modeling the cell in three dimensions.

It is worth noting that according to the calculations here, surrounding cells accounted for an average of 65% of the dissected volume. In contrast, about 50% of two dissected R2 cells measured by Berry and Cohen (1972) were glial in volume. (This figure was obtained by working backwards through the line labeled "control^3" in their Table 2. Similarly, when their Figure 2a, a section of a dissected R2, was analyzed according to the model used in this chapter, the surrounding cells accounted for 50% of the volume of the dissected unit.) Furthermore, in doing the present work, four other R2 cells which had not been labeled were dissected, sectioned, and stained. In these cells, cells surrounding the neuron accounted, respectively, for 30%, 55%, 40%, and 45% of the total volume of the dissected unit. Figure 4 shows a section of the cell which was estimated to be about 30% glial in volume. The relatively high surrounding cell volume in the cells which were autoradiographed is another reason for assuming that the figures given actually overestimate the usual amount

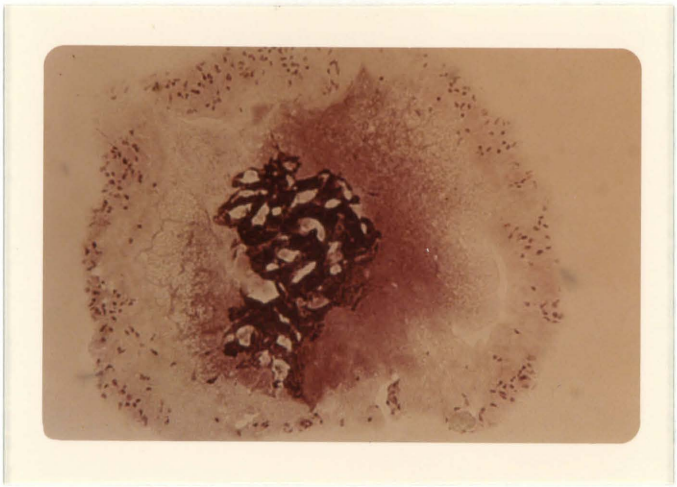


Figure 4. Section of a dissected R2.

of contamination encountered.

In considering SDS polyacrylamide gel patterns, we may speculate that proteins of contaminating cells have as broad a distribution of molecular weights as the neuronal pattern, i.e., generally<20% of total counts at any one molecular weight. The following calculation can be made:

(a) total contaminating counts in the sample: generally < 20% of total counts in the entire sample.
(b) in the pattern of contaminating counts, the maximum counts at a particular molecular weight: generally < 20% of the total counts in the entire pattern.

Therefore: maximum contamination at a particular molecular weight:
generally < 20% x 20% = < 4% of total counts in the entire sample.

Decreases in contaminating cells can be no greater than this. If increases are also no greater, then localized changes in gel patterns greater than 4% of the total control pattern are likely to be largely neuronal in origin. In the previous chapter it was noted that the mean increase in percent total counts at 50K for the three cells showing a high $[K^+]$ effect was 4.5%. Since this was an increase of 4.5% which occurred while total incorporation was decreasing 35%, it really represented only 3% of the total control counts, which is below the 4% level which I have suggested as critical. It therefore remains possible that the K^+ effect on incorporation at 50K has its origin in the glia. A more definitive answer must await the use of microdissection techniques, such as those of Kato and

Lowry (1973), by which glial cells may be accurately separated from neuronal cell bodies.

In contrast, the decrease in total incorporation into giant cell caused by high [K^+] must be at least partially neuronal in origin. The average decrease in incorporation was 35%, whereas the maximum decrease attributable to contaminating cells would be around 20%.

SUMMARY

Localization of ^{3}H-leucine incorporated into parieto-visceral ganglia of Aplysia californica was investigated with autoradiography. Studies in whole ganglia confirmed Strumwasser's previous observation that the grain density over nerve cell bodies was much higher than over glial cells. Grain density over the cytoplasm of the giant cell (R2) averaged 14 times the grain density over adjacent patches of glial cells. The neuropil region contained few, if any, grains above background.

The relative contribution of contaminating cells (mostly glia) to the total incorporation of dissected neurons was studied in sections of cells dissected from labeled ganglia. Grain densities in neuronal and contaminating regions of the "cell" were counted on several sections, and a geometrical model was used to compute the relative contribution of these regions to the whole "cell." Both the assignment of grains to the two regions and the

geometrical model tended to overestimate the relative contribution of the contaminating cells. According to these calculations, contaminating cells accounted for 11% and 34% of the incorporation into two R2 cells and 12% and 16% of the incorporation into two R15 cells. In considering incorporation patterns observed on SDS polyacrylamide gels, the following speculation was offered: The maximum contamination at a particular molecular weight was unlikely to be more than 4% of total counts. Changes larger than this are, therefore, likely to be neuronal in origin.

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CHAPTER V

AUTORADIOGRAPHY OF PLEURO-VISCERAL CONNECTIVE NERVE

As reported in Chapter II, high $[K^+]$ (90 mM $[K^+]$) media caused an 85% decrease in incorporation into pleuro-visceral connective nerves. These nerves contain a considerable amount of connective tissue sheath. In the work reported in this chapter autoradiography was used to determine the distribution of incorporated label between connective tissue and the axonal region (containing glial cells) of the nerve, and to determine the distribution of the high $[K^+]$ effect on incorporation.

MATERIALS AND METHODS

PVGs were dissected from the animal as usual (pleuro - visceral connective nerves were cut about midway between the PVG and the pleural ganglia). PVGs went through two 90 minute rinses in blood salts media and then a 240 minute incubation in media containing ^{3}H-leucine. At the end of incubation, ganglia went through two 10 minute rinses in blood salts media at 0^oC, after which pleural-visceral connective nerves were cut free and sectioned as described below. Media were either normal media or experimental media containing 90 mM $[K^+]$ throughout (contents of media given in Table I, Chapter II).

Pleuro-visceral connective nerves from a normal incubation and a high [K^+] incubation were placed parallel to one another on a block of Tissue-Tek Embedding Medium (Ames Company, Elkhart, Indiana) on a heat sink in an American Optical cryostat having an ambient temperature of -23°C. The tissue was covered with another layer of Tissue Tek and the whole block was embedded in Tissue Tek on the microtome stage so that the axes of the nerves were perpendicular to the cutting plane of the knife. Thus, a normal section and an experimental section were cut in a single section and were located close to one another when the section was picked up on a warm slide. Sections were dried, fixed, rinsed, autoradiographed, stained, and counted as described in Chapter IV. About half of the sections which were analyzed were counted "blind" by a person[1] who did not know the purpose of the experiment or the identity of the sections. (Note: only a few sections for each pair of nerves were counted. These few were the ones which retained the best morphology. Many of the sections not counted were ripped or distorted rather badly. It is felt, on the basis of later experience with other tissues that better sectioning would have been achieved if nerves had first been dipped briefly in liquid Tissue Tek before being placed on the frozen Tissue Tek block. The

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[1]Joe Carlsen, an undergraduate at Caltech.

knife apparently cuts more uniformly through tissue which has been so treated.)

RESULTS AND DISCUSSION

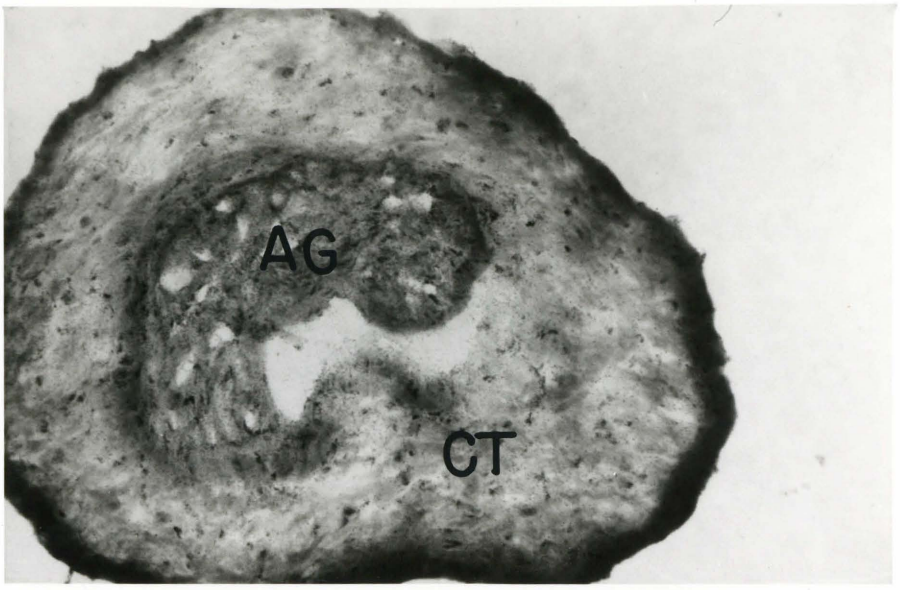
Figure 1 shows autoradiographs of nerve sections obtained from a normal and a high $[K^+]$ incubation. Results obtained from sections of 3 pairs of nerves are tabulated in Tables Ia and Ib.

Incorporation in connective tissue was not uniform. Grain density was usually higher around cell nuclei stained in the connective tissue. In contrast, the axonal-glial region of the nerve had a relatively uniform density of grains.

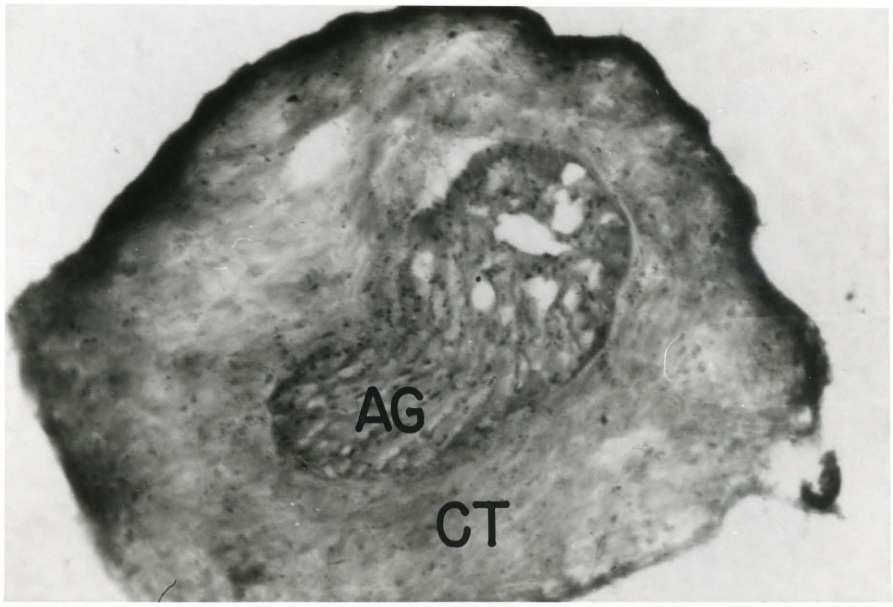
In both the normal incubation and the high $[K^+]$ incubation the connective tissue sheath accounted for approximately 70% of the total incorporation in the nerve, though the proportion in connective tissue tended to be somewhat higher in high $[K^+]$ incubations (Table Ib, last column).

High $[K^+]$ caused a drop in the grain density over both connective tissue and axonal-glial regions of the nerve. The mean ratio of total grains in normal nerve to total grains in nerve incubated in high $[K^+]$ was 5.1, which would correspond to an 80% decrease in incorporation. This corroborates the previous observation of a decrease in leucine incorporation caused by high $[K^+]$ incubation.

Figure 1. (a) autoradiograph of cross-section of a pleuro-visceral connective nerve which was labeled with ^{3}H-leucine in normal medium. (b) Autoradiograph of cross-section of a pleuro-visceral connective nerve which was labeled with ^{3}H-leucine in medium containing 90 mM [K^{+}]. This nerve was frozen in the same block as the nerve in (a). The sections shown in (a) and (b) were sectioned at the same time and are located next to one another on the same slide. (c) higher power magnification of part of (a). (d) higher power magnification of part of (b). CT, connective tissue sheath; AG, axonal-glial region. These sections are 172,24,1 and 173,24,2 (see Table I).



(a)



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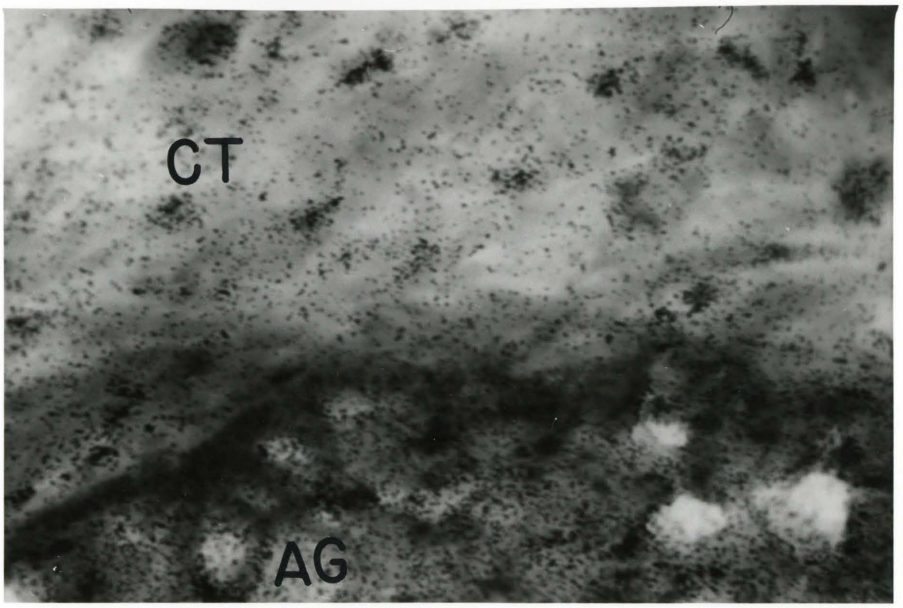

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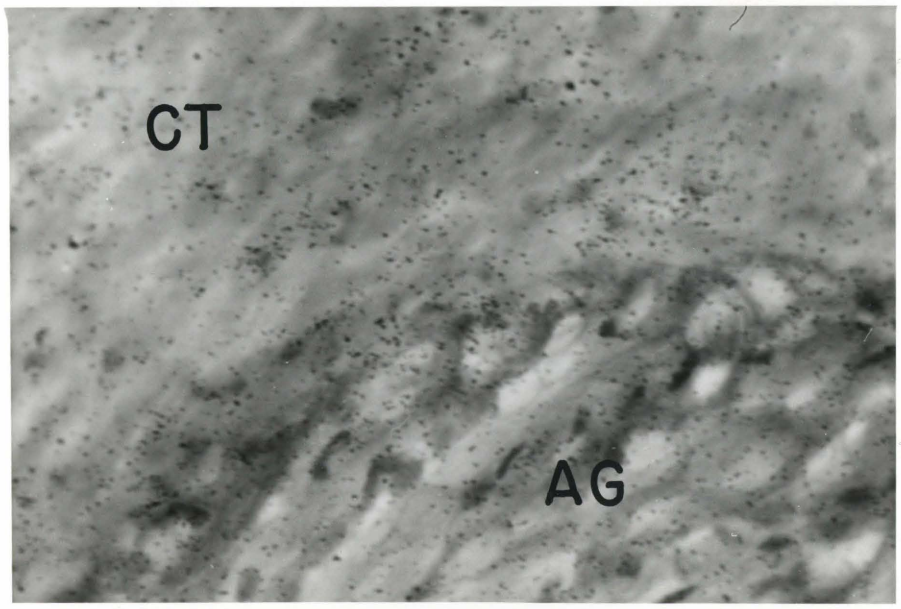

(d)

TABLE I. (being a summary of Tables Ia. & Ib.)

AUTORADIOGRAPHY OF PLEURO-VISCERAL CONNECTIVE NERVE

Nerve pair (inc. 10 mM K^+, inc. 90 mM K^+)	Ratio of grain densities (10 mM K^+: 90 mM K^+)		Ratio of total grains in nerve (10 mM K^+: 90 mM K^+)
	connective tissue sheath	axonal-glial region	
168,169	5.3	3.2	4.5
170,171	9.4	7.2	6.2
172,173	3.8	8.8	4.6
mean	6.2	6.4	5.1

	Per cent of total grains in nerve appearing in connective tissue sheath	
10 mM K^+	64 ± 6%	not significantly different*
90 mM K^+	74 ± 15%	

*t test for paired variates

TABLE Ia.

AUTORADIOGRAPHY OF PLEURO-VISCERAL CONNECTIVE NERVE

a. GRAIN DENSITIES

Sample				Connective tissue sheath			Axonal-glial region		
animal	slide	section	$[K^+]$ (mM)	grain density[1] (grains/69 μ^2)		ratio of grains $\frac{10\ mM}{90\ mM}$	grain density[1] (grains/69 μ^2)		ratio of grains $\frac{10\ mM}{90\ mM}$
168	54	1	10	24.8	21.6		39.7	38.7	
168	54	3	10	18.4		4.0	37.8		3.0
169	54	2	90	5.4			12.9		
168	54	4	10	18.7	17.1		38.4	34.8	
168	54	6	10	15.6		7.1	31.2		3.6
169	54	5	90	2.4			9.7		
168	54	7	10	16.8	18.0		39.3	33.4	
168	54	9	10	19.3		4.8	27.4		2.9
169	54	8	90	3.8			11.7		
	mean					5.3			3.2
170	28	3	10	52.0		4.6	75.1		6.5
171	28	1	90	11.2			11.6		
170	28	9	10	68.1		5.2	108.9		7.9
171	28	7	90	13.1			13.7		
170	38	1	10	40.6		18.4	67.3		7.1
171	38	3	90	2.2			9.5		

TABLE Ia. (CONTINUED)

mean (170,171)				9.4		7.2	
animal	slide	section	$[K^+]$ (mM)	grain density	ratio	grain density	ratio
172	10	1	10	20.9	3.5	50.0	6.8
173	10	2	90	5.9		7.3	
172	10	3	10	33.0	5.3	77.5	6.2
173	10	4	90	6.2		12.6	
172	24	1	10	17.1	2.5	53.6	13.4
173	24	2	90	6.9		4.0	
mean					3.8		8.8
MEAN of the means					6.2		6.4

[1]Background has been subtracted.

TABLE Ib.

AUTORADIOGRAPHY OF PLEURO-VISCERAL CONNECTIVE NERVE

b. TOTAL GRAINS

				connective tissue			axonal-glial			total cross section		
animal	sample slide	section	$[K^+]$ (mM)	A area (mm^2)	B^1 total grains ($x10^{-2}$)		C area (mm^2)	D^1 total grains ($x10^{-2}$)		B+D total grains ($x10^{-2}$)	ratio of total grains 10 mM / 90 mM	fraction of grains in conn. tissue
168	54	1	10	.230	822	584	.062	355	282	866	3.5	.67
168	54	3	10	.130	345		.039	210				
169	54	2	90	.239	186		.034	63		249		.75
168	54	4	10	.210	566	465	.049	273	223	688	5.7	.68
168	54	6	10	.162	364		.039	173				
169	54	5	90	.206	71		.035	49		120		.59
168	54	7	10	.213	515	506	.046	263	202	707	4.3	.71
168	54	9	10	.178	496		.036	140				
169	54	8	90	.227	124		.023	39		163		.76
	mean										4.5	
170	28	3	10	.124	930		.043	469		1369	5.3	.66
171	28	1	90	.128	206		.035	58		264		.78
170	28	9	10	.142	1389		.067	1049		2438	6.0	.57
171	28	7	90	.153	288		.059	117		405		.71

TABLE Ib. (CONTINUED)

animal	slide	section	$[K^+]$	connective tissue area	connective tissue total grains	axonal-glial area	axonal-glial total grains	total cross section total grains	total cross section 10 mM / 90 mM	total cross section fraction in conn. tissue
170	38	1	10	.134	782	.034	327	1109	7.2	.71
171	38	3	90	.218	69	.063	86	155		.45
	mean								6.2	
172	10	1	10	.220	662	.049	356	1018	4.5	.56
173	10	2	90	.228	194	.033	34	228		.93
172	10	3	10	.254	1206	.062	693	1899	5.6	.63
173	10	4	90	.288	257	.045	81	338		.76
172	24	1	10	.274	675	.057	437	1112	3.6	.61
173	24	2	90	.283	281	.056	32	313		.90
	mean								4.6	
	MEAN of means								5.1	

[1]equal to the area times the grain density (TABLE Ia).

SUMMARY

Localization of ^{3}H-leucine incorporated into pleuro-visceral connective nerves and the effect of high $[K^{+}]$ media on this incorporation was studied using autoradiography. About 70% of the total incorporation in the nerves was found in the connective tissue sheath. Incorporation in high $[K^{+}]$ media was reduced to about 20% of normal level in both the connective tissue sheath and the axonal-glial region of the nerve.

CHAPTER VI

GENERAL DISCUSSION

In this discussion my intention is first to review the most important results and conclusions of this thesis, noting the limits and shortcomings of the data supporting them. Subsequently, I would like to deal with the question of what further experiments could be undertaken to learn more about these phenomena and their possible biological roles.

REVIEW OF RESULTS

Incubation of the parieto-visceral ganglion in high $[K^+]$ (90-110 mM $[K^+]$, with equimolar $[Na^+]$ reduction) media caused a relative increase in incorporation of leucine into aqueous soluble peptides of approximately 50K (K = 1000 daltons) molecular weight. High $[K^+]$ caused a decrease in total incorporation into the ganglion and, in general, a greater decrease into high molecular weight peptides. With "reduced Na^+, normal K^+," none of these effects occurred, thereby suggesting that the effects were due to the raised $[K^+]$, and not the lowered $[Na^+]$. A possible, though not favored, alternative is that $tris^+$, which was added instead of K^+ in this control, was blocking a "low Na^+" effect. High $[K^+]$ incubation also caused a change in the fraction

of labeled protein which was aqueous soluble. This change in relative solubility was, however, too small to have produced the relative increase seen at 50K in the aqueous soluble fraction. The change in incorporation at 50K may reflect the change in synthesis of a specific protein.

A number of experiments indicated that the relative increase in incorporation at 50K had occurred in the neurons: (a) a relative increase at 50K did not occur when pleuro-visceral connective nerves, which contain connective tissue and glial cells but no neuronal cell bodies, were incubated in high $[K^+]$, and (b) a relative increase at 50K was observed in R2 cells which were dissected from ganglia incubated in high $[K^+]$ media.

Nevertheless, the neuronal origin of this relative increase at 50K is not certain. The dissected R2 experiments were plagued by a great deal of variability. Moreover, an autoradiographic study of R2 showed that enough incorporation took place in the adhering glial cells so that the effect at 50K could have had its origin in them.

The decrease in total incorporation of leucine into PVG protein, which occurred during high $[K^+]$ incubation, took place both in neurons and connective tissue. The 35% decrease observed in dissected R2 cells was too large to be explained solely by decreases in adhering glial cells. Connective tissue sheath from which cell bodies had been scraped showed a 57% decrease in incorporation.

An even more remarkable decrease in incorporation in high [K^+] media was observed in pleuro-visceral connective nerve. Incorporation decreased to only about 15% of normal levels. Autoradiographic studies showed that incorporation in the nerve decreased both in its connective tissue sheath and in its axonal region, where presumably most of the synthesis was glial. Non-volatile TCA soluble label in the nerve and the PVG did not change appreciably in high [K^+] incubation, and therefore no support is given to the idea that these changes were due to a decrease in precursor specific activity.

FURTHER EXPERIMENTS

There are a number of weak spots in the data which additional experiments might help strengthen. First, it would be desirable to obtain a more reliable result with dissected cells. Stabilizing the solubilization conditions by increasing ionic strength and adding carrier proteins would be useful; however, in view of the partially positive results with the aqueous insoluble fraction, perhaps the most straightforward approach would be simply to homogenize the cell in SDS buffer and run the whole SDS soluble fraction.

Second, the question of whether the effect is really neuronal in origin is still not decisively answered. Running microdissected regions of sections of dissected

cells on SDS gels should yield the information needed.

There are a number of questions which would be worthwhile asking, particularly if the high $[K^+]$ effect on 50K peptides(s) can be shown to be neuronal in origin. These are: (a) what is the nature and function of the 50K peptide(s), and (b) what is the mechanism responsible for its relative increase in high $[K^+]$ media?

With the first question, we are more or less in a shot in the dark situation; however, there are some hints worth investigating. The molecular weight of the peptide of interest is near that of tubulin. Moreover, the pleuro-visceral nerve shows a large 50K staining peak though it synthesizes comparatively little peptide at this molecular weight. Thus the 50K peptide may well be transported from neuronal cell bodies through the nerve, and may indeed be tubulin, which is thought to play a role in axonal transport. Vinblastine precipitates tubulin as well as a few other proteins (Barondes & Feit, 1970; Wilson, et al., 1970), and has been used as a criterion for identifying tubulin in brain tissue (Barondes & Feit, 1970). I would propose (1) that the molecular weight of tubulin in Aplysia be determined by running SDS gels of vinblastine precipitates (previously done for vertebrate brain by Barondes & Feit, 1970), and (2) that if it co-chromatographs with the Aplysia 50K peak, the effect of high $[K^+]$ on the relative amount of incorporation into this

precipitate be investigated. With further information lacking, it could only be a matter of speculation as to why synthesis of tubulin or any other particular protein might increase in response to high $[K^+]$ incubuation.

With regard to the mechanism of the relative increase in incorporation at 50K, questions can be asked at two loci: (1) the protein synthesis machinery and (2) sites of direct action by $[K^+]$. It is my personal opinion that looking at the protein synthesis end is a fruitless approach. Presently, we know too little about what controls protein synthesis in any eukaryotic cell to presume to investigate it in an expensive and specialized tissue like the Aplysia nervous system. The experiments with $[K^+]$ were originally undertaken to determine what specific neuronal characteristics might play a role in modulating protein synthesis. Rather than using high $[K^+]$ and attempting to block one or another of the possible consequences of high $[K^+]$ treatment (such as using ouabain to block active transport or low $[Ca^{++}]$ to block transmitter release), as was suggested in the discussion in Chapter II, a more valuable strategy would be to choose those consequences of high $[K^+]$ which can be produced by more physiological stimuli and test whether these more physiological stimuli may be able to reproduce some of the effects caused by high $[K^+]$. This would have the advantage that whatever effects are produced, whether similar to the high $[K^+]$ effect or not, would be more

likely to have some importance in the normal functioning of the nervous system. For example, to test whether synaptic stimulation by transmitter released by high $[K^+]$ may have been responsible for the high $[K^+]$ effect, the Wilson and Berry (1972) stimulation experiment should be repeated using the internal control protocol employed in this thesis. I believe that the internal control procedure is sufficiently more sensitive than the parallel control method used by Wilson and Berry (1972) to make this a worthwhile experiment.

Another question of interest is what is the mechanism responsible for the decrease in total incorporation caused by high $[K^+]$ media. The particularly large effect seen in the nerves and their relative abundance and uniformity in Aplysia make them a choice preparation for investigating this question further. Heald (1960) has suggested a central role of ion transport in this phenomenon in vertebrate brain slices (see Introduction to this thesis), yet no one has reported the effect of ouabain, an inhibitor of ion transport, on this high $[K^+]$ effect. I would propose testing the effect of ouabain on the high $[K^+]$ effect on total incorporation into pleuro-visceral connective nerve.

Finally, it would be highly relevant to know whether there are situations in the Aplysia nervous system in which external $[K^+]$ increases to a point where the

results of these high [K^+] experiments are directly applicable. In the Introduction to this thesis, it was noted that Orkand, et al. (1966) had observed large increases of external [K^+] due to repetitive stimulation of single neurons and nerve roots. They could stimulate Necturus nerve until it was blocked, presumably by build-up of external [K^+]. Glial cells in the nerve were depolarized by an amount corresponding to an external [K^+] concentration of 20 mM K^+. This must be less than the actual level to which [K^+] had increased adjacent to axons since not all membranes of the glial cell face axons. To determine what level of [K^+] may have been present in the nerve when conduction was blocked, they tested the effect of known levels of K^+ on nerve conduction. 30 mM [K^+] always blocked, and 23 mM [K^+] did not. Thus repetitive stimulation of Necturus nerve led to the increase in external [K^+] in some parts of the nerve to around 25 mM, a level which is about 8 times the normal Ringer concentration of 3 mM. A comparable increase in Aplysia, which has a normal external [K^+] of 10 mM, would be to 80 mM. Inasmuch as effects on total incorporation in Aplysia nerve were observed at 50 mM [K^+], it seems possible that some of the effects reported in this thesis may reflect phenomena that occur in vivo, albeit probably on a more localized scale. Without further experimentation, this is sheer speculation. I would therefore propose testing whether

Aplysia pleuro-visceral connective nerve can be blocked by repetitive stimulation, and if so, what levels of K^+ can block conduction. Such an experiment, modeled after Orkand, et al. (1966), would give us an indication of whether the levels of K^+ used in this thesis, and the phenomena accompanying them, may have a direct bearing on the functioning of neurons and glial cells in the nervous system of Aplysia.

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APPENDIX A

MOLECULAR WEIGHT SCALE OF SODIUM DODECYL SULFATE POLYACRYLAMIDE GELS

The molecular weight of bands stained on SDS gels used in this work has been determined by two methods: (1) comparing ganglion samples of marker proteins of known molecular weight (MW) run on separate gels and (2) running ganglion samples with marker proteins added. The procedure for the former method is as follows: At the end of electrophoresis the length of the gel and the position of the tracking dye, bromphenol blue, were noted. Gels were then stained, destained, and traced as described in Chapter II. The mobility of a band on a gel relative to the tracking dye was calculated as

$$\text{Mobility} = \frac{\text{distance of band on tracing}}{\text{length of tracing of gel}} \times \frac{\text{gel length before staining}}{\text{distance of dye migration before staining}}$$

Table I shows the mobilities of marker proteins and bands a, b, c, d, and e in the ganglion pattern relative to the tracking dye (for identification of bands see Figure 2a).

A least squares plot of log(MW) **v. relative mobility** for the marker proteins was made (Figure 1), and the positions of the average relative mobilities of bands a, b,

TABLE I.

MOBILITY OF MARKER PROTEINS AND PVG PROTEINS

RELATIVE TO TRACKING DYE

Marker protein[1]	BSA	γG-H	Oval	Chym	γG-L[2]	cyt c
Molecular weight[1]	68K	52K	45K	26K	24K	11.7K
mobility ± standard deviation	.565 ± .019	.617 ± .022	.704 ± .018	.921 ± .007	.865 ± .006	1.091 ± .009
(N)	(4)	(6)	(6)	(3)	(3)	(3)

PVG protein band[3]	a	b	c	d	e
mobility ± standard deviation	.665 ± .030	.729 ± .034	.787 ± .033	.876 ± .036	.953 ± .038
(N)	(20)	(20)	(20)	(20)	(20)

[1]Abbreviations and molecular weight references are as follows: BSA, bovine serum albumin, Castellino & Barker, 1968; γG-H, human gamma globulin, heavy chain, Edelman, et al., 1968; Oval, ovalbumin, Castellino & Barker, 1968; Chym, bovine chymotrypsinogen A, Dayhoff, 1972; γG-L, human gamma globulin, light chain, Edelman, et al., 1968; cyt c, horse heart cytochrome c, Dayhoff, 1972.

[2]In addition to the three cases indicated, γG-L was also present in the three gels containing Chym. Chym was present in much greater amount than γG-L, and only one band could be distinguished.

[3]Identification of bands is shown in Figure 2a. Bands a, b, and c are the same as those indicated in Chapter II, Figure 2.

Figure 1. A least squares plot of log(MW) v. mobility of marker proteins relative to the bromphenol blue tracking dye. Molecular weights and identities of the markers which were used are given in Table I.

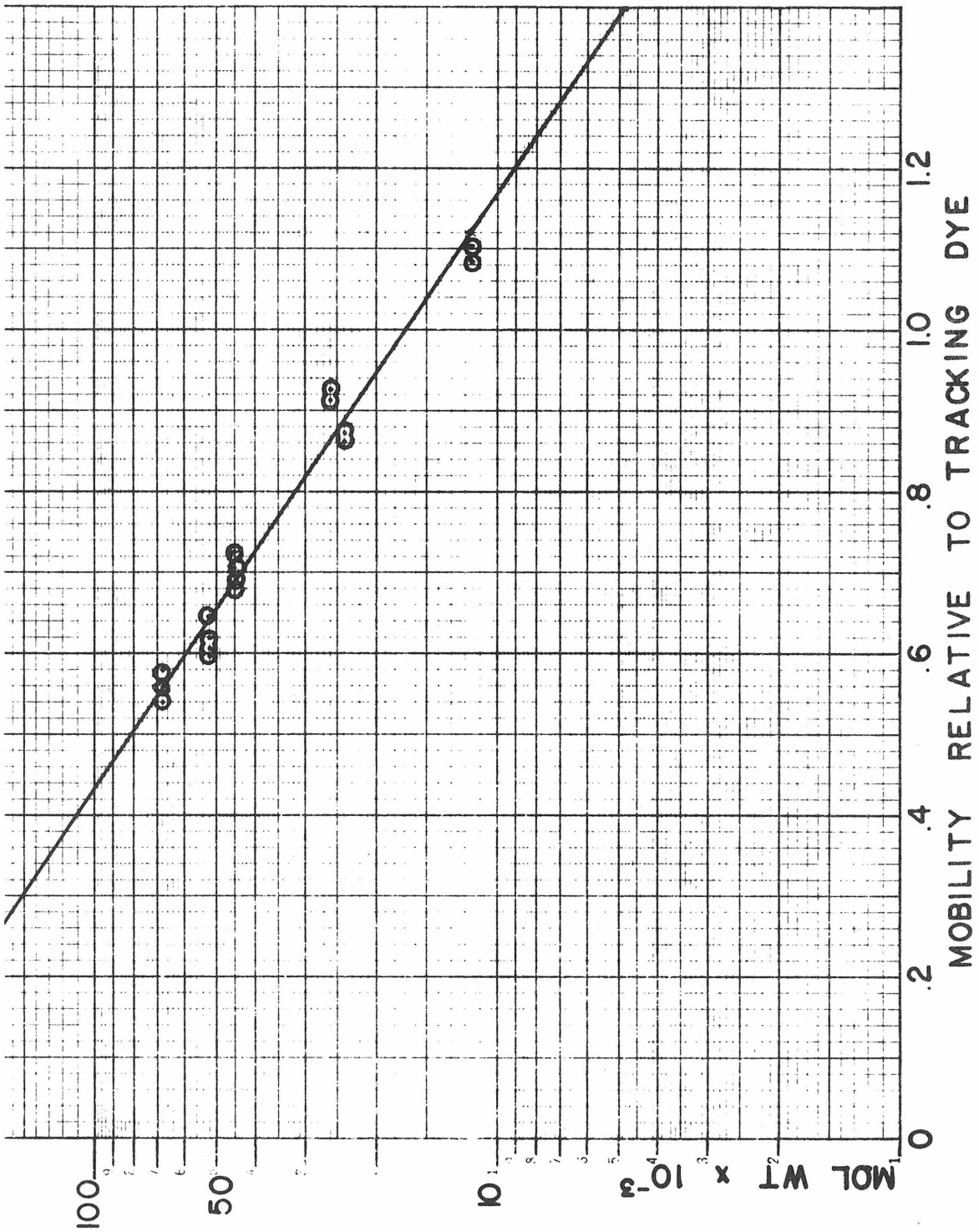
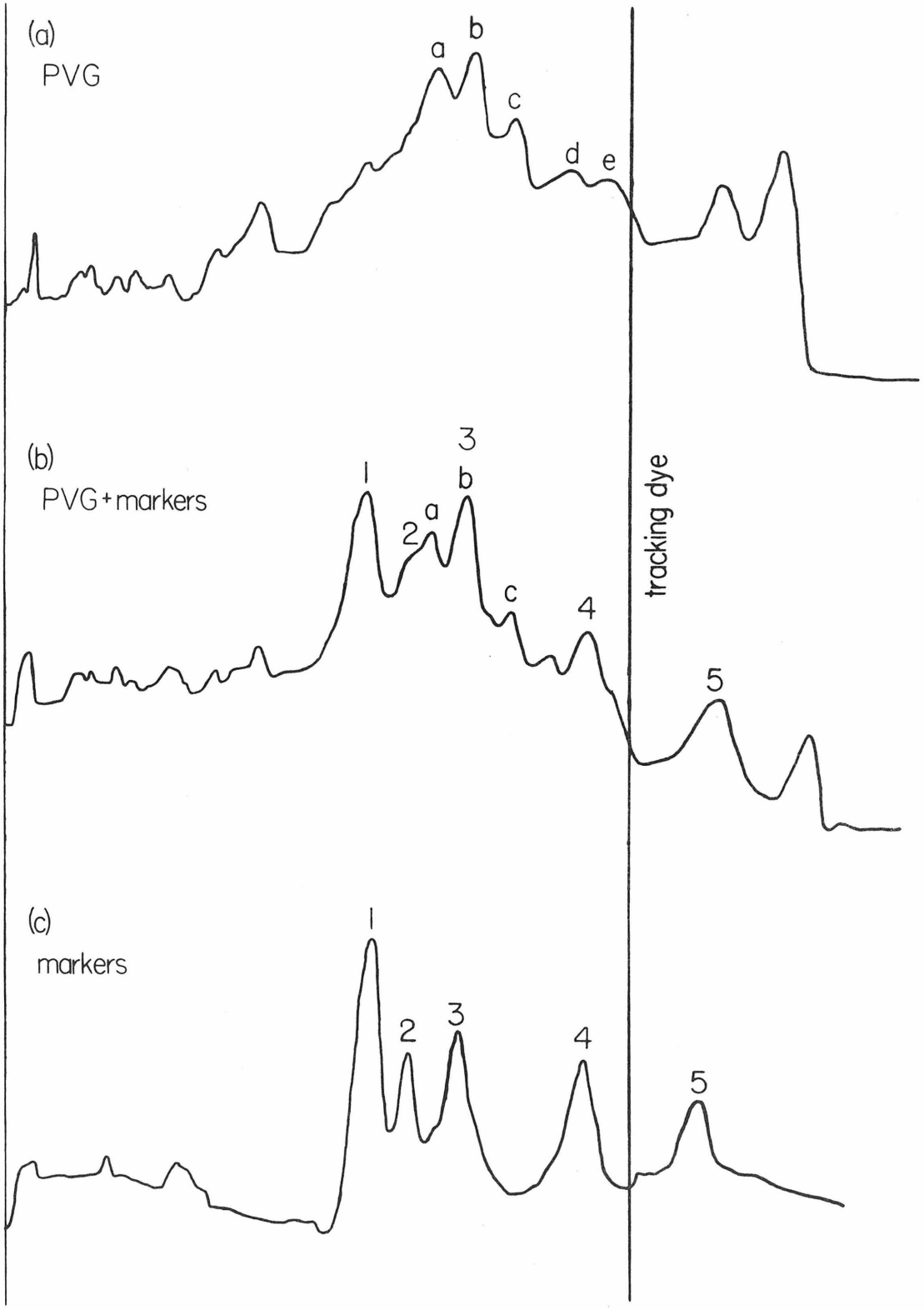

MOL WT x 10^{-3}
100
50
10
0
.2
.4
.6
.8
1.0
1.2
MOBILITY RELATIVE TO TRACKING DYE

Figure 2. (a) normal staining pattern of a PVG. Several consistently seen bands in the pattern are identified as bands a, b, c, d, and e. Bands a, b, and c are the same as those indicated in Chapter II, Figure 2. (b) staining pattern produced when a combination of marker proteins was added to a sample of the PVG shown in (a). The marker proteins alone produced the pattern shown in (c). The marker proteins are the following: 1, bovine serum albumin; 2, human gamma globulin, heavy chain; 3, ovalbumin; 4, mostly chymotrysinogen A, with a small amount of human gamma globulin, light chain; and 5, horse heart cytochrome c. Molecular weights and references are given in Table I.


(a)
PVG
a
b
c
d
e
(b)
PVG + markers
1
2
a
3
b
c
4
5
tracking dye
(c)
markers
1
2
3
4
5

c, d, and e were located. Determined by this method, the molecular weights of a, b, c, d, and e are 48.3K, 39.6K, 33.1K, 25.1K, and 19.8K, respectively (K = 1000 daltons).

Figure 2b shows the pattern produced when a combination of marker proteins (Figure 2c) was added to a sample of the aqueous soluble fraction of the PVG (Figure 2a). Band a ran between the H-chain of human gamma globulin (MW = 52,000 (Edelman, et al., 1968)) and ovalbumin (MW = 45,000 (Castellino and Barker, 1968)). Positions of marker proteins and band c relative to band a are given in Table II. Band b cannot be distinguished from ovalbumin (MW = 45,000). A least squares plot of the marker proteins was made (Figure 3). Locating bands a and c on this plot gives molecular weights of 49.8K and 33.8K respectively.

Weber and Osborn, 1969 have reported on the reliability of SDS polyacrylamide gel electrophoresis. The maximum variability of about 5% which I observed in mobility relative to the tracking dye (Table I) is in agreement with the 5-10% deviation of mobility observed by Weber and Osborn for proteins run on separate occasions. The maximum deviation observed by Weber and Osborn of molecular weight determined on SDS polyacrylamide gels from molecular weights determined by other methods was nearly 10% in the 10,000 to 70,000 molecular weight range. Given this possible amount of error, the molecular weights in the text

TABLE II.

MOBILITY OF MARKER PROTEINS

AND BAND C RELATIVE TO BAND A[1]

Marker or band[2]	BSA	γG-H	Chym	γG-L	cyt c	band c
Molecular weight[2]	68K	52K	26K	24K	11.7K	---
Run 77	.846	.944	1.34	---[3]	1.65	1.17
Run 82	.864	.960	1.38	---[3]	1.69	1.20
Run 96	.860	.962	---	1.37	---	1.19
MEAN	.857	.955	1.36	1.37	1.67	1.19[4]

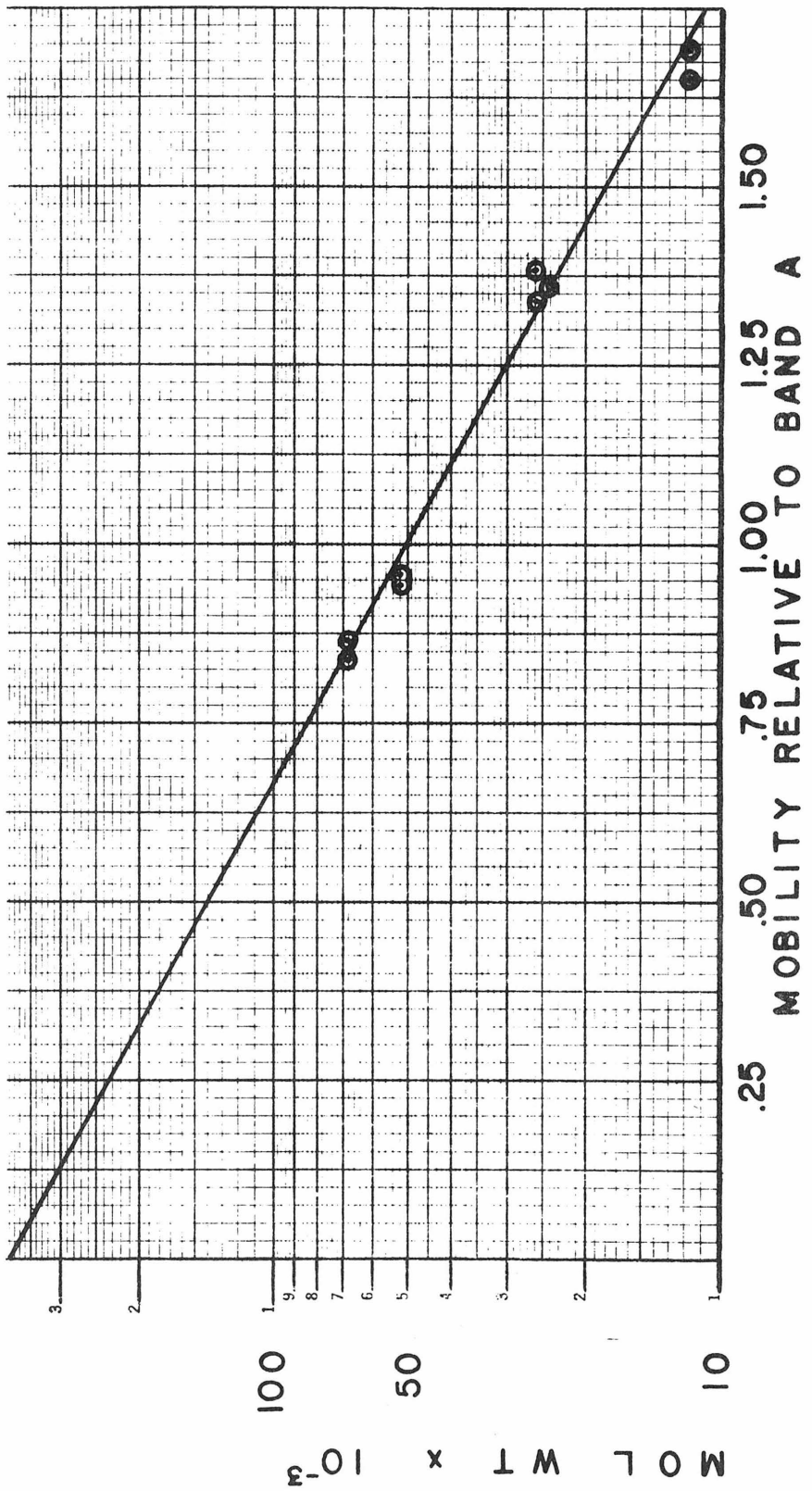
[1]Solutions of marker proteins have been added to samples of the aqueous soluble fraction of the PVG.

[2]Abbreviations and molecular weight references given in TABLE I.

[3]γG-L is present here but in much smaller quantity than Chym. These two proteins cannot be differentiated.

[4]By way of comparison, the mean mobility of band c relative to band a on the 20 gels reported in TABLE I. was 1.18.

Figure 3. A least squares plot of log(MW) v. mobility of marker proteins relative to band a for experiments in which marker proteins were added to PVG samples. Molecular weights and identities of the markers which were used are given in Table I.


MOL WT x 10^{-3}
100
50
10
.25
.50
.75
1.00
1.25
1.50
MOBILITY RELATIVE TO BAND A

of this thesis have been reported only to the nearest 5000 daltons. Bands a, b, and c are reported as 50K, 40K and 35 K respectively.

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APPENDIX B

CONCERNING THE RATIO OF ^{3}H COUNTS TO ^{14}C COUNTS

In Chapter II, several experiments were done with a simultaneous incorporation procedure, in which a parieto-visceral ganglion (PVG) was labeled simultaneously with ^{3}H-leucine and ^{14}C-leucine. These experiments showed that the incorporation pattern obtained with ^{3}H-leucine was essentially the same as that obtained with ^{14}C-leucine. In doing these experiments, however, it has been noticed that the ratio of ^{3}H counts to ^{14}C counts in the labeled protein was lower than was expected.

The ratio of counts expected can be computed as follows:

$$\text{ratio of counts} = \frac{\text{amount of } ^{3}H \text{ in incubation medium} \times {}^{3}H \text{ counting efficiency}}{\text{amount of } ^{14}C \text{ in incubation medium} \times {}^{14}C \text{ counting efficiency}}$$

$$= \frac{\frac{1}{2} \times 50\ \mu C \times .4}{\frac{1}{2} \times 2.5\ \mu C \times .75} = 10.1$$

The factors of $\frac{1}{2}$ in both the numerator and denominator are due to the fact that in this protocol only $\frac{1}{2}$ of the usual amount of each label is used in the incubation medium. This number actually varied somewhat (perhaps $\pm$ 10%) depending on the counting efficiency of the counter and

variation in the specific activity of the labeled compound as supplied by the manufacturer. In the four simultaneous incorporation experiments reported in Chapter II the following procedure was used: Ten µl aliquots of incubation medium were taken following incubation and counted in 10-20 ml of scintillation fluid containing NCS (Ward, et al., 1970). The ratios of counts in this aliquot and in gels on which the incubated tissue was run were calculated after correcting for background and channel overlap. Results are shown in Table I.

Another two simultaneous incorporation experiments were done in which the tissue was analyzed according to the procedure shown in Figure 12, Chapter II; that is, the labeled PVG was separated into aqueous soluble and aqueous insoluble fractions which were precipitated and washed in ice-cold 10% TCA and then counted directly in scintillation fluid containing NCS. 25 µl aliquots of the TCA soluble counts of these ganglia were counted both directly in scintillation fluid and after being dried for several hours at 60° C. 10 µl aliquots of the incubation media were likewise counted both directly and after being dried for several hours at 60° C. Results of these experiments are shown in Table II.

DISCUSSION

The results show, with only one exception, that

TABLE I. RATIO OF COUNTS, $^{3}H:^{14}C$

SIMULTANEOUS INCORPORATION EXPERIMENTS

Sample	Ratio of counts, $^{3}H:^{14}C$	
	on gel	in 10 ul incubation medium
PVG 97-sol.	4.6	10.1
PVG 97-insol.	4.6	
PVG 129-sol.	5.9	5.7
PVG 129-insol.	5.8	
PVG 156-sol.	7.8	10.6
PVG 157-sol.	7.8	11.0

PVG 156 and PVG 157 were incubated on the same day.
$[K^{+}]$ for PVG 156 was 90 mM and for 157 was 10 mM.
$[K^{+}]$ for all other experiments was 10 mM.

TABLE II. RATIO OF COUNTS, $^{3}H:^{14}C$

SIMULTANEOUS INCORPORATION EXPERIMENTS

sample	aq sol TCA insol	aq insol	TCA sol		incubation medium	
			direct	dried	direct	dried
PVG 1	5.1	5.1	6.4	6.2	9.6	7.5
PVG 2	5.1	5.1	6.6	6.3	9.4	7.4

PVGs labeled in simultaneous incorporation experiments had lower ratios of ^{3}H to ^{14}C than did a sample of the medium in which they were incubated. The single exception showed a lower ratio of counts in the medium than was expected according to the calculation made at the beginning of this Appendix.

These results suggest that the label in the ^{3}H-"leucine" was not in the same compounds that the label in ^{14}C-"leucine" was in. Some of the label in the ^{3}H-"leucine" was apparently in compounds which were not so readily incorporated into the PVG as the ^{14}C-"leucine," hence a lower ^{3}H:^{14}C ratio in the PVG than in the medium.

The experiments reported in this Appendix used ^{3}H-leucine from at least three different shipments; therefore, a simple case of mislabeling by the manufacturer is probably not responsible. The similarity of the gel patterns produced by the ^{3}H and ^{14}C labels also suggests that the two labels have much in common; i.e. they probably do contain leucine, though this has not been checked independently by chromatography.

A part of the difference may be due to $^{3}H_2O$ in the ^{3}H-leucine. This is indicated by the decrease in incubation medium ^{3}H:^{14}C ratio when the medium was dried (Table II). However, this does not explain the discrepancy completely. The ratio in the PVG in these experiments was still 30% less than the dried incubation medium ratio.

These facts are considered in relation to the experiments reported elsewhwere in this thesis:

(1) Is most incorporation into protein? The impurities present in the ^{3}H-leucine might be labeling other compounds.

Wilson (1971), using incubation media very similar to those used here, showed that puromycin, a protein synthesis inhibitor, blocked most ^{3}H-leucine incorporation into higher molecular weight molecules (i.e. slower moving bands) in neuronal cell bodies and inhibited total incorporation by 77%. Gainer (1972) and Schwartz, et al. (1971), using simpler media (not much more than ^{3}H-leucine in a physiological saline), also showed that most of the label is probably in protein. Gainer (1972) digested labeled samples with the proteolytic enzymes in pronase, ran them on SDS gels, and found few counts above background. Schwartz, et al. (1971) showed that anisomycin, another protein synthesis inhibitor, inhibited total incorporation by over 95% and also showed that pronase digestion decreased TCA insoluble label by 95%. Therefore, it seems likely that most incorporation observed on the gels used in this thesis is label incorporated into protein.

(2) Is high $[K^{+}]$ producing its effects by affecting the metabolism of some impurity in the ^{3}H-leucine rather than affecting the incorporation of leucine into

protein?

Several experiments suggest that high [K^+] is <u>not</u> exerting its effects by affecting some impurity in the ^{3}H-leucine. Two experiments described in Chapter II show that the high [K^+] effect at 50K is independent of whether the label being incorporated is ^{3}H or ^{14}C. Furthermore, PVG 156-sol. and PVG 157-sol., which were incubated on the same day and thus received aliquots of the same label and were electrophoresed and counted together, showed identical ^{3}H:^{14}C ratios. The former was incubated in 90 mM [K^+] and the latter in 10 mM [K^+].

The conclusion, therefore, is that whatever may be the cause of decreased ratios of ^{3}H:^{14}C in simultaneous incorporation experiments, this cause probably does not greatly affect the results obtained in the main body of this thesis.

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APPENDIX C
WHAT IS THE MEANING OF "RELATIVE INCREASE"?

Does saying that a relative increase in incorporation occurred at 50K give us any information about whether an increase in the absolute amount of incorporation into some peptide at 50K has occurred? In the context of the data given in this thesis, the answer to this question is no. This is due to the fact that the total incorporation into all peptides in the PVG decreased by 50% (see p. 84) while the percent of total counts at 50K increased by 50% (see Table IV, Chapter II). Thus, the total amount of incorporation at 50K under high K^+ conditions actually decreased to 75% of the normal level of incorporation (a 50% increase above a 50% level of incorporation brings the level of incorporation up to 75%).

If incorporation at 50K were primarily into a single peptide, then "relative increase" in incorporation would mean that the 50K peptide had decreased in incorporation to only 75% of its normal level while most other peptides had decreased to 50% of their normal level.

On the other hand, a peptide species at 50K comprising only 10% of normal 50K incorporation might be the only one not to show the "usual" 50% decrease in incorporation. In that case the "relative increase" in incorporation at 50K would mean that incorporation into this particular species had actually increased to 300% of

its normal level while total incorporation at 50K fell to 75% of its normal level. This is calculated as follows:

> 90% of normal incorporation undergoes 50% decrease: yields 90% x 50% = 45% of normal level.
>
> The remaining 30% of normal level (i.e. 75% - 45%) is due to the species ordinarily comprising only 10% of normal incorporation.
>
> An increase from 10% to 30% of normal incorporation is a 300% increase.

Thus, it is clear that without further information regarding what species and what fraction of total 50K incorporation are "relatively increased" by high K^+, we cannot say whether this change represents an absolute increase or a lesser decrease in incorporation. Such further information might be forthcoming if attempts to separate components located at 50K are tried.