

SPECIFIC GLYCINE UPTAKE BY IDENTIFIED NEURONS OF *APLYSIA CALIFORNICA*. II. BIOCHEMISTRY

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(Accepted January 18th, 1978)

SUMMARY

Glycine is taken up twice as rapidly by neurons R3-R14 as by other identified neurons in the *Aplysia* parieto-visceral ganglion. Earlier studies had shown that R3-R14 have much higher glycine concentrations than other *Aplysia* neurons. Most of the glycine taken up by R3-R14 was biochemically untransformed for at least 1 h following its uptake. Glycine is actively transported into R3-R14 and other *Aplysia* neurons by carrier-mediated processes. Glycine uptake by R3-R14 was markedly reduced in the absence of Na^+ and in the presence of Hg^{2+} , while these treatments had little effect on glycine uptake by other *Aplysia* neurons. There appears to be a special glycine uptake system present in R3-R14 and a general glycine uptake system common to all *Aplysia* neurons. The elevated glycine concentrations and special glycine uptake associated with R3-R14 may indicate that glycine is utilized as a neurotransmitter by those neurons.

INTRODUCTION

The cell bodies of the *Aplysia* neurons R3-R14 were more intensely labeled than those of their neighbors in autoradiographs of ganglia incubated in [^3H]glycine (see accompanying report)²⁴, demonstrating that R3-R14 take up glycine relatively rapidly. We here describe the characterization of glycine uptake by R3-R14 and other *Aplysia* neurons by radiochemical means.

MATERIALS AND METHODS

Animals and Materials

Animals and chemicals used are described in the accompanying report²⁴.

Determination of uptake into cells

The uptake of amino acids by the *Aplysia* parietovisceral ganglion (PVG) neurons was characterized by incubating isolated ganglia in small vials containing 0.1–0.5 ml of solutions of the labeled amino acids in artificial seawater at 25 °C. Two or more animals were subjected to each treatment. The values from separate ganglia were combined to give the reported values for R3-R14 and other neurons. The non-R3-R14 neurons examined consisted approximately of R2, R15, L2-L6 and L11. A mixture of labeled and unlabeled glycine was used at the highest concentrations employed. Isotonicity was maintained in the Na⁺-dependence experiments by replacing NaCl with choline chloride. The effects of mercuric chloride, 2,4-dinitrophenol and ouabain were tested by preincubating ganglia in solutions of those compounds in artificial seawater followed by incubations in [³H]glycine solutions containing those substances. Incubation vials were cooled to 4 °C by placing them in an ice bath to determine the effect of temperature on glycine uptake. The uptake of [³H]alanine was characterized by 20 min incubations in 3.7 μM [³H]alanine.

Following incubation, ganglia were rinsed in artificial seawater and fixed for 2 h in 3.2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.4 M sucrose and 0.072 M CaCl₂. This procedure gave about a 50% higher recovery of radioactivity from individual cells than did dissection from unfixed ganglia. Individual cell bodies were identified on the basis of size, location and pigmentation as described by Frazier and coworkers⁹. They were dissected from the ganglia, cell volumes determined as described below, and the cell bodies transferred to scintillation vials. Following incubation overnight at 40 °C in 0.5 ml of tissue solubilizer (Protosol, New England Nuclear), 5 ml of Aquasol (New England Nuclear) and 0.1 ml of 4.0 M acetic acid were added to each vial. Samples were then counted for 5 min each in a Packard Tri-Carb liquid scintillation spectrometer. Samples consisting of tissue solubilizer and acetic acid were used as blanks. The quantities of glycine taken up were determined by comparing sample counts per minute to the counts per minute from known amounts of [³H]glycine.

Cell volume determinations

Cell volumes were determined after the cells were freed from the ganglia by measuring maximum cell diameters and diameters perpendicular to the maximum diameters by means of either a calibrated ocular micrometer or a microscale placed adjacent to the cells. Cell volumes were calculated using the formula for an oblate spheroid: $V = 4\pi ab^2/3$, where a is half the maximum and b is half the second diameter¹¹. The theoretical validity of this method of determining cell volumes was checked by determining the dimensions of the cell bodies of 4 neurons from the R3-R14 group and of 2 other cells using 115 × photographs of 8 μm serial sections. The volumes were calculated on the basis of the formula described above using the two longest of the 3 cell body diameters. The volumes of the same cells were then redetermined by using a planimeter to measure the areas of the examined cells in each section in which they appeared, multiplying the section areas by the section thicknesses to get cell volumes per section, and summing the volumes per section. Cell volumes

based on the latter measurements should have been accurate within a few per cent. Volumes calculated on the basis of the diameters were 2.0 times those based on section volumes for R3-R14, and 1.6 times the section volumes for other cells. Therefore, the concentrations reported may be low by perhaps a factor of 2. However, any errors should have been similar for R3-R14 and other cells, and so would not significantly affect the comparisons of the two types of cells.

Chemical characterization of glycine transport into neurons

A PVG from a 250 g animal was incubated for 1 h in 500 μ l of artificial seawater containing 4.4 nmole of [3 H]glycine. Cells were then dissected from the ganglia and placed in one of 3 pools consisting of R3-R14, other cells or bag cells. The cell bodies from each pool were ground into the same spot on a sheet of Whatman No. 1 filter paper and unlabeled glycine applied as a carrier to each spot. The chromatograms were developed with *n*-butanol:glacial acetic acid:water (12:3:5; v/v/v) and then scanned with a Packard 7201 radiochromatogram scanner. Since this chromatographic system did not resolve glycine and serine, additional analyses were carried out by thin layer chromatography (TLC). Tissues and single cells were each extracted with 200 μ l of 70% ethanol for 18 h. The ethanol extracts were dried in a vacuum centrifuge and the residues taken up in 10 μ l of 70% ethanol. The ethanol solutions and standard amino acids were applied to TLC plates (type LQDF, Quantum Industries), the chromatograms developed with *n*-butanol:acetone:diethylamine:water (20:20:3:10; v/v/v/v), and the amino acids visualized with ninhydrin. The distribution of radioactivity on the plates was determined by passing the plates through a radiochromatogram scanner and by scraping 1.0 cm segments of adsorbent from the plates into scintillation vials, adding 0.5 ml of water and 5.0 ml Aquasol (New England Nuclear) and counting in a scintillation spectrometer. The ethanol-insoluble residues were digested with protosol and counted to determine the fraction of [3 H]glycine incorporated into macromolecules.

RESULTS

Glycine uptake by Aplysia neurons

R3-R14 took up glycine about twice as fast as other neurons over wide ranges of time, concentrations and temperature (Table I, Figs. 2 and 3). Representative values for one neuron from each group were R14: $45 \pm 14 \mu$ M and R2: $16 \pm 9 \mu$ M (from 3 ganglia incubated in 8.8 μ M [3 H]glycine for 20 min). Similar relative rates of [3 H]glycine uptake were obtained at all times examined, and at external glycine concentrations up to 140 μ M. None of the non- R3-R14 neurons had uptake rates consistently in the R3-R14 range. The rates of glycine uptake derived for neurons in the same group varied several fold in the same animal, and average uptake rates of corresponding groups of cells in this study varied by as much as a factor of 2 between animals. An evaluation of the significance of the differences in glycine uptake between R3-R14 and the other neurons by statistical means such as the *t*-test was not possible, since the variances of the two sets of data were significantly different³.

TABLE I

Conditions affecting glycine uptake

Ganglia were preincubated for the specified times under the given treatments and then incubated under the same conditions for 20 min in 8.8 μM [^3H]glycine under the treatment conditions. Values are \pm S.E.M.

Condition	R3-R14		Other neurons	
	No. Cells	Uptake (μM)	No. Cells	Uptake (μM)
Control	12	32 \pm 5	23	16 \pm 2
Removal of Na ⁺ (5 min)	14	7.2 \pm 1.3	14	11 \pm 2
Mercuric chloride (0.1 mM, 5 min)	14	14 \pm 2	15	11 \pm 2
Mercuric chloride (0.1 mM, 60 min*)	12	37 \pm 7	11	46 \pm 13
Control (60 min*)	24	76 \pm 11	38	32 \pm 3
2,4-Dinitrophenol (1 mM, 30 min)	13	32 \pm 4	14	17 \pm 3
Ouabain (0.1 mM, 30 min)	14	26 \pm 4	14	13 \pm 1.3
4 °C (5 min)	10	21 \pm 6	13	9.8 \pm 1.4

* Total incubation time.

Chemical characterization of [^3H]glycine following its uptake

The radioactivity in the cell bodies of R3-R14 and of other neurons was found by paper chromatography to be over 90% glycine plus serine (the two amino acids were not separated by the solvent system used) and less than 10% macromolecular (Fig. 1). However, about 50% of the radioactivity in the bag cells remained at the origin (Fig. 2), probably due to incorporation into the neurosecretory peptides manufactured by those cells^{2,10,28}. The levels of radioactivity in the ethanol insoluble residues of the cell bodies extracted for TLC analyses confirmed that about 50% of the glycine taken up was incorporated into protein in the bag cells, and that only 10% was so incorporated in other neurons. However, about 20% of the radioactivity in R3-R14 and about 30% of the radioactivity in the other neurons from ganglia incubated in [^3H] or [^{14}C]glycine for 1 h comigrated with serine. Less than 4% of the radioactivity in all cells from ganglia incubated for 1 h in [^3H]serine was in glycine. Approximately 2/3 of the tritium present in the L[G- ^3H]serine used in this experiment would have been lost in the transformation of serine to glycine. Therefore as much as 12% of the serine taken up by Aplysia neurons could have been converted to glycine.

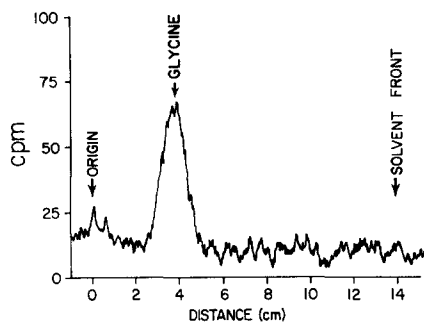


Fig. 1. Scan of a paper chromatogram of the radioactivity in R3-R14 following incubation in [^3H]glycine-containing seawater. For conditions, see text.

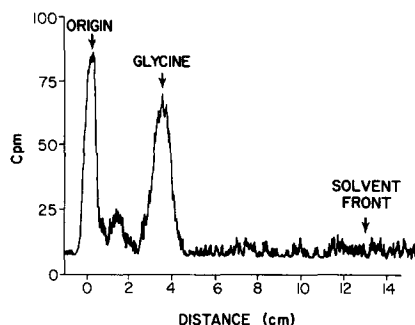


Fig. 2. Scan of a paper chromatogram of the radioactivity in the neurosecretory bag cells following incubation in [^3H]glycine-containing seawater. The large peak at the origin is probably due to incorporation into peptides. For conditions, see text.

Alanine and leucine uptake

Intracellular alanine concentrations reached $0.50 \pm 0.06 \mu\text{M}$ in R3-R14 and $0.30 \pm 0.03 \mu\text{M}$ in other neurons in 2 ganglia incubated for 20 min in $3.4 \mu\text{M}$ [^3H]-alanine. The corresponding values for 2 ganglia incubated for 20 min in $8.8 \mu\text{M}$ [^3H]-leucine were $19 \pm 8 \mu\text{M}$ and $26 \pm 14 \mu\text{M}$.

Time dependence

Uptake by both groups of cells was nearly linear for the first hour with marked curvature occurring at longer times (Fig. 3). A standard incubation time of 20 min was chosen for other experiments under the assumption that this was long enough that rates of uptake into cell bodies should not have been substantially influenced by the rates of transport into the ganglion, while still being in the approximately linear region of the time dependence curve.

Concentration dependence

The concentration dependences of glycine uptake by R3-R14 and other neurons are presented in Fig. 4 as S/V vs S plots based on the form $[S]/v = [S]/V_{\text{max}} + K_m/V_{\text{max}}$ of the Michaelis-Menton equation. This form was utilized instead of $1/V$ vs $1/S$ plots, as the uncertainties in the values of the intercepts in the latter plots caused a very large uncertainty in the resulting values of V_{max} and K_m . The lines drawn through the points were determined by least-squares calculations using the mean uptake values for each concentration. The correlation coefficients were 0.75 for the R3-R14 plot and 0.90 for the other neurons. The kinetic constants derived for the neurons other than R3-R14 were K_m , $1400 \mu\text{M}$ and V_{max} , $0.15 \mu\text{mole/g-min}$. The corresponding values for R3-R14 were $2200 \mu\text{M}$ and $0.300 \mu\text{mole/g-min}$.

Na^+ dependence

When 80% or more of the NaCl in the incubating solutions was replaced with choline chloride, uptake by R3-R14 was reduced by almost 80% in 20 min incubations, while a smaller reduction may have occurred in the other neurons (Table I).

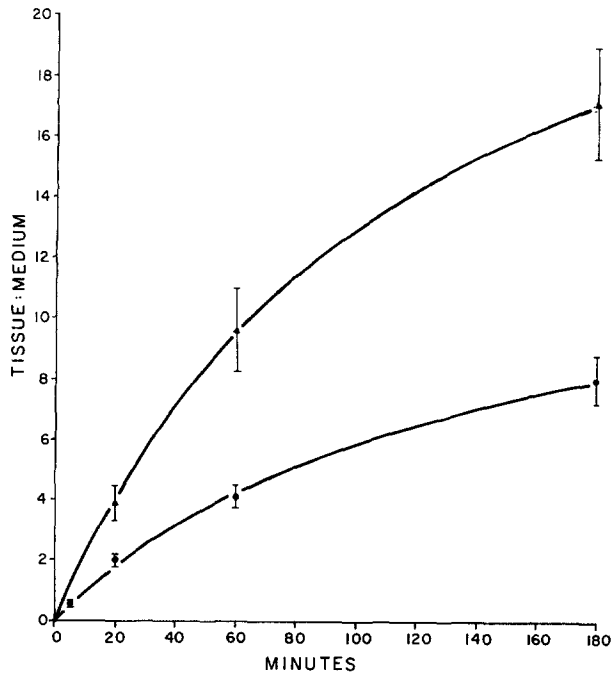


Fig. 3. Time dependence of [^3H]glycine uptake by *Aplysia* neurons. Triangles, R3-R14; circles, other neurons. Incubation conditions are described in the text. The error limits are \pm S.E.M.

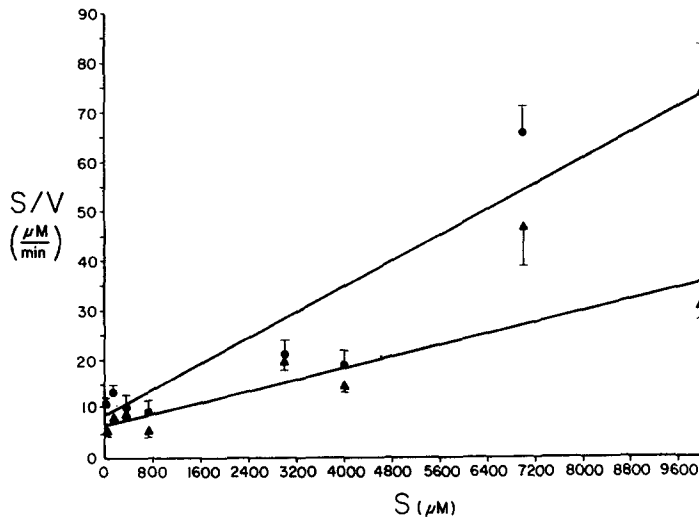


Fig. 4. S/v versus S plots of the dependence of [^3H]glycine-uptake by *Aplysia* neurons on external glycine concentrations. Triangles, R3-R14; circles, other *Aplysia* neurons. Correlation coefficients were R3-R14, 0.75; other neurons, 0.90.

The effects of chemical agents and temperature

Incubation of PVG in 0.1 mM HgCl₂ caused no clear change in [³H]glycine uptake by most neurons, while reducing uptake by R3-R14 twofold. Preincubation for 30 min in 0.1 mM ouabain caused at most slight reductions in uptake by either type of cell. Five and 30 min preincubations in 1.0 mM solutions of 2,4-dinitrophenol followed by standard incubations in [³H]glycine-containing solutions that were 1.0 mM in 2,4-dinitrophenol had no detectable effect on glycine uptake. Reducing the temperature to 4 °C caused about a one-third reduction in the rate of uptake into all neurons.

DISCUSSION

Glycine uptake by identified Aplysia neurons

This study confirms the autoradiographic demonstration²⁴ that the Aplysia neurons R3-R14 take up glycine more rapidly than their neighbors. About twice as much radioactivity was found in R3-R14 as in other neurons following incubation in [³H]glycine solutions in this study, while the number of grains over the cell bodies of R3-R14 in autoradiographs were 3–4 times higher than over the cell bodies of other neurons²⁴. The source of this discrepancy is unknown.

Elevated rates of glycine uptake and unusually high intracellular glycine concentrations are demonstrated to be properties of the same neurons by this and the accompanying report²⁴. Since glia as well as neurons specifically take up putative neurotransmitters¹² including glycine²⁵, it is generally not certain which tissue elements are responsible for the uptake processes studied in slices of nervous tissue. However, high glycine concentrations and high affinity glycine uptake systems are associated with the same areas of the vertebrate nervous system^{18,21}.

Specificity of glycine uptake

Alanine, the amino acid chemically most similar to glycine, also appeared to be taken up preferentially by R3-R14, though it was taken up about 20 times more slowly than glycine. However, this result is contradicted by the fact that autoradiographs of ganglia incubated in alanine had comparable numbers of grains over R3-R14 and other neurons²⁴. Leucine and serine were taken up at comparable rates of R3-R14 and other neurons, and serine, leucine and proline labeled R3-R14 and neighboring neurons to almost identical degrees in autoradiographs²⁴. Therefore, R3-R14 selectively take up glycine.

Biochemistry of glycine taken up by Aplysia neurons

Except in the bag cells, which manufacture neurosecretory polypeptides, most of the glycine taken up by Aplysia neurons does not undergo rapid biochemical transformation. A fraction, which is comparable in all Aplysia PVG neurons, is converted into what is probably serine. Serine taken up by Aplysia neurons was more slowly converted to glycine than glycine to serine. Serine is thought to be the biochemical precursor of glycine in the vertebrate nervous system^{1,27}.

Characterization of glycine uptake

Since tissue: medium concentration ratios well above 1 are achieved by uptake of glycine into both R3-R14 and other *Aplysia* neurons (Fig. 2), glycine must be actively transported into both types of cells. If the glycine which is taken up is exposed to all of the intracellular glycine immediately after entering the cells, then glycine uptake into *Aplysia* neurons can occur against concentration gradients of at least 1000:1, since glycine concentrations in *Aplysia* neurons are 1 mM and higher¹⁵. Evidence that this is probably the case will be discussed below.

Glycine uptake by the non-R3-R14 neurons obeys Michaelis–Menton kinetics (Fig. 3), indicating that glycine is taken up by those cells by a carrier-mediated transport system. The K_m of this system is about 1400 μM , while the K_m derived for R3-R14 is 2200 μM . K_m 's are about 100 μM for low affinity glycine uptake and about 10 μM for high affinity glycine uptake in vertebrate nervous tissue¹⁸. Thus the general glycine uptake system is a low affinity system in comparison to the glycine uptake systems present in vertebrate neural tissue. If there are two distinct glycine uptake systems in R3-R14 (see below), then the kinetic characteristics of the systems are similar enough and the uncertainty in the data great enough that the kinetic constants for the two systems cannot be derived.

Removal of Na^+ or the presence of Hg^{2+} drastically slowed the rate of glycine uptake into the R3-R14, but had little if any effect on glycine uptake by other neurons. Our results seem to indicate that in the absence of Na^+ , glycine is taken up slightly less rapidly by R3-R14 than by other *Aplysia* neurons (Table I). In an autoradiographic experiment, R3-R14 were 1.5 times as intensely labeled as other neurons in the absence of Na^+ , while the corresponding ratio was 3–4 in autoradiographs of ganglia incubated under standard conditions²⁴. Thus glycine is taken up at similar rates by R3-R14 and most other *Aplysia* neurons in the absence of Na^+ . Ouabain, a Na^+/K^+ ATPase inhibitor, and 2,4-dinitrophenol, an inhibitor of oxidative phosphorylation, had little effect, even after 30 min preincubations. Therefore, glycine uptake by R3-R14 depends on the presence of a Na^+ gradient, but is not directly coupled to the activity of the Na^+ pump. The Na^+ gradient probably persists for a long time across the membrane of the large *Aplysia* neurons following inhibition of Na^+ transport, as the Na^+ gradient across the membrane of the squid giant axon dissipates very slowly following treatment with ouabain, though the Na^+ pump is inactivated almost immediately by treatment with ouabain^{3,13}. The squid giant axon takes up both glycine and glutamate at normal rates for 40–50 min following ouabain treatment, while removal of external Na^+ inhibits the uptake of glutamate, but has little effect on glycine uptake⁵. Ouabain, 2,4-dinitrophenol, Hg^{2+} and the removal of Na^+ inhibit high affinity glycine uptake in vertebrates^{21,22}, while low affinity glycine transport is not absolutely dependent on Na^+ (see ref. 29). By analogy to the characteristics of amino acid uptake by the squid giant axon, we interpret our data as indicating that there is a general glycine uptake system common to all *Aplysia* neurons that is relatively insensitive to Na^+ and an additional Na^+ dependent system which is specific to R3-R14. Uptake by the two types of cells should be affected to the same proportion by removal of Na^+ if only one system exists in all of the neurons and R3-R14 posses higher concentrations of carriers

in their cell membranes. The general system may be slightly Na^+ dependent. Systems that are only partially dependent on Na^+ are known^{19,20}.

High affinity uptake systems in vertebrates and high affinity choline uptake in *Aplysia* are both strongly temperature sensitive⁸. However, glycine uptake by both the white and non-white cells was only slightly temperature dependent. Since the white cells are electrically active at temperatures as low as 3 °C (see ref. 23), they may have an uptake system that is relatively insensitive to temperature.

Uptake as a source of intracellular glycine

The average glycine concentrations are 5 mM in R3-R14 and 1 mM in the other neurons¹⁴. Tissue: medium ratios of 17:1 in R3-R14 and 8:1 in the other neurons are attained in 3 h (Fig. 2). Since the glycine concentrations in the *Aplysia* hemolymph is about 32 μM (see ref. 16) and the rate of glycine uptake is a nearly linear function of concentration between 8.8 and 32 μM , glycine levels in the intact animal would reach 540 μM in R3-R14 in 3 h by uptake, and 250 μM in 3 h in other neurons. Therefore about 11 % of the intracellular glycine in R3-R14 and about 25 % of that in the other neurons would be derived by uptake in that time. The net accumulation of [³H]glycine in the final 2 h of incubation is about equal to that in first hour in both types of cells. If this rate of decline in net glycine accumulation can be extrapolated to longer times, it would take about a day for the amounts of glycine accumulated by uptake to equal the endogenous levels, assuming that the rates of conversion of glycine to other substances at longer times are similar to those derived in this study. Therefore uptake from the extracellular medium can probably supply all of the glycine present in *Aplysia* neurons. This may contrast with the situation in the rat CNS, where it has been concluded that most of the glycine present is obtained by de novo synthesis from serine²⁷.

Net glycine accumulation by uptake in both R3-R14 and other neurons probably slows as the rates of transport out of the cells become significant relative to the rates of uptake. Similar decreases in the rate of accumulation of labeled amino acids with time are commonly observed in samples of vertebrate nervous tissue^{16,21}. This is to be expected, as active transport systems are capable of transporting their substrates in both directions across cellular membranes⁶. However, glycine also leaves cell bodies of R3-R14 by axonal transport²⁴.

Function of glycine uptake by R3-R14

In conclusion, the identified *Aplysia* neurons R3-R14 appear to have a specific uptake system for glycine that enables them to take up glycine from the extracellular medium several times as rapidly as their neighbors. Specific amino acid uptake systems present in vertebrate nervous systems are thought to be indicative of neurotransmitter functions for the compounds taken up²⁰. They serve to remove released neurotransmitter molecules from synapses and help to maintain presynaptic stores of transmitters^{16,29}. As the probable sites of neurotransmitter release from R3-R14 are some distance from the cell bodies of R3-R14, uptake by those cell bodies probably does not serve to inactivate glycine following its release as a neurotransmitter. Since glycine

taken up into the cell bodies of R3-R14 is transported into their axons²⁴, glycine may be transported down the axons of R3-R14 to be released as a neurotransmitter. There is precedent for this possibility, since there is strong evidence that glycine acts as a neurotransmitter in the vertebrate spinal cord¹.

ACKNOWLEDGEMENTS

This work was supported by DHEW grant NS 12567 and Robert A. Welch Foundation grant H-609 to DJM. We also wish to acknowledge the technical assistance of Mr. Roy Okuda and helpful discussions with Dr. R. E. Coggeshall and Dr. J. S. Kittredge.

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