and sulfuric acids (1 : 1). The sample was washed and the nitrated cellulose leached out with nine 200-ml portions of acetone; four more than required to obtain a clear solution.

The carbon and minor inorganic matter remaining in the filter were aerated with an aspirator; it was washed with distilled water, and dried in an oven. The carbon was converted to carbon dioxide and analyzed for radiocarbon in the standard manner. The radiocarbon activity observed, less than 0.55 percent of modern (*4*) and equivalent to an apparent age of more than 40,000 years, clearly demonstrates that plant contaminants can be completely removed from charcoal samples by the described chemical treatment.

To test the unlikely possibility that insoluble organic compounds might form in the acetone-nitrocellulose reaction and be absorbed by the charcoal, 3.95 g of young charcoal which was dated as being 1280 ± 130 years old (A-601) was contaminated with 5.93 percent of grass roots (A-487) as shown in Fig. 1 and purified in the same way as before. Ages calculated in two separate counts were 1300 ± 300 and 1410 ± 260 years which were averaged to 1360 ± 200 years (A-718). Because the difference between this result and that of the uncontaminated sample is approximately one-third of the standard deviation of the difference, there is 87 percent probability that the two samples have the same C^{14} content.

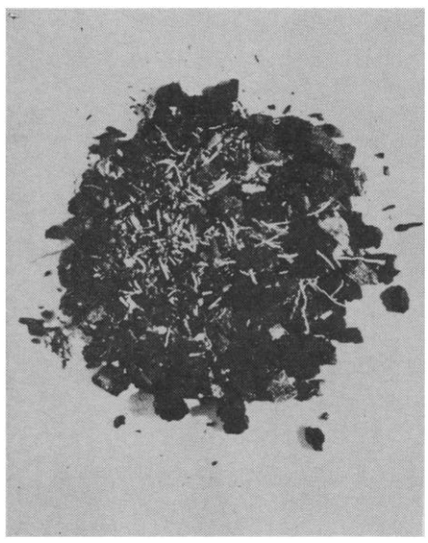

Fig. 1. Charcoal sample A-601 showing its appearance after contamination with 5.93 percent of modern grass roots (A-487). The pile is approximately 8 cm in diameter.

Therefore, the acetone has no significant effect on the C^{14} age of the sample.

This purification treatment, while suitable only for elemental carbon (*5*), is recommended for all charcoal samples suspected of being near the age limit of radiocarbon detection. Its application to very old samples requiring isotopic enrichment would appreciably improve the accuracy of the dating.

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5. Our results, and presumably those of J. G. Ogden, III, and R. J. Hay, *Radiocarbon* **7**, 172 (1965), show that there is a considerable loss of sample if it is only partially carbonized, such as carmelized wood or lignite.
6. Contribution No. 108 of the University of Arizona's program in geochronology. Sponsored by NSF (grant GP-2330). The assistance of R. Bennett and D. C. Grey in the laboratory was much appreciated, as were comments on the manuscript by P. E. Damon and R. Berger.

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Internally Perfused Axons: Effects of Two Different Anions on Ionic Conductance

Abstract. *Voltage-clamped giant axons of squid, internally perfused with potassium chloride solutions, showed reduced initial transient membrane conductance to voltage and increased overall (leakage) conductance. Unclamped axons showed reduced action and resting potentials. Ionic conductances and membrane potentials were maintained or restored by perfusion with potassium fluoride solutions. As much as 90 percent of internal fluoride could be replaced with chloride without alteration of normal properties of membrane.*

The survival time of internally perfused giant axons of squid, in terms of resting potential, action-potential magnitude, and excitability, depends on, among other things, the type of anions contained in the perfusion solution (*1*). Our results now extend these findings by applying the voltage-clamp technique to study of the perfused giant axon.

The technique for simultaneous voltage-clamping and internally perfusing isolated, cleaned, giant axons of squid (*Loligo pealii*) has been described (*2*). Membrane potentials were measured with a glass microelectrode (3*M* KCl bridge connected to a calomel half cell) inserted just through the membrane for recording the potential with respect to an external glass-millipipette electrode (3*M* KCl-agar bridge connected to a calomel half cell). The electrodes were shorted together in 0.5*M* KCl between experiments; there was always less than 1-mv junction potential between them. No correction was made for additional junction potential after penetration of the membrane with the microtip electrode, because junction potentials of less than 1 mv were recorded between the two electrodes by the method of Baker, Hodgkin, and Shaw for various external- and internal-perfusion solutions (*3*).

Internal-perfusion flow rates were about 10^{-4} ml/sec. Internal solutions were changed by replacing one Sage electrolytically driven pump with another containing a different solution; the dead space of the cannula was exchanged within 2 minutes. The external-perfusion solution, artificial sea water, had the following ion concentrations (m*M*): Na^+, 430; K^+, 10; Ca^{++}, 10; Mg^{++}, 50; Cl^-, 560; tris buffer, 1. The first of three internal solutions had the following ion concentrations (m*M*): K^+, 456.6; F^-, 400; $HPO_4^=$, 26.6; $H_2PO_4^-$, 3.4. Potassium chloride replaced KF in the second solution, and the third contained nine parts of the KCl solution and one part of the KF solution. The *p*H of all solutions was between 7.2 and 7.4.

The presence of 26.6-m*M* $HPO_4^=$ in our solutions, although richer than the concentration used by Tasaki *et al.* (*1*), probably did not obscure the other anion effects studied; in most instances it was only 1/15 of the concentration of anions being tested.

After change of the internal solution from 400-m*M* KF to 400-m*M* KCl, the resting potential, E_{RP}, declined about 10 mv in 15 minutes while the action-potential peak voltage, E_{AP}, fell quite low. After 10 minutes of KCl perfusion the rate of action-potential decrease accelerated; rise in threshold to excitation

was about tenfold during this final 5-minute period. These results resemble those of Tasaki *et al.* (*1*) and typify our results with ten different preparations of axons. Survival times of excitability of axons internally perfused with the 400-m*M* KCl solution varied between 10 and 45 minutes, in contrast with times as long as 4 hours with 400-m*M* KF solution.

Figure 1 illustrates the typical change, in membrane current in the voltage clamp, associated with this anion effect. Initial internal perfusion was with 400-m*M* KF solution; the upper record in *A*, obtained with this solution, shows the maximum transient inward current following a step change in membrane potential from a 50-msec prepulse potential of −105 mv to a pulse potential of −5 mv. Whenever sodium is the major cation in external perfusion, this transient inward component of membrane current is assumed to be carried by sodium ions (*4*); we shall call the component a sodium current. The lower record in Fig. 1*A* shows the membrane current corresponding to a hyperpolarizing step in membrane potential from the holding potential of −57 mv (equal to the resting potential just before voltage clamping) to a pulse potential of −120 mv; the step current is about 0.1 ma/cm^2.

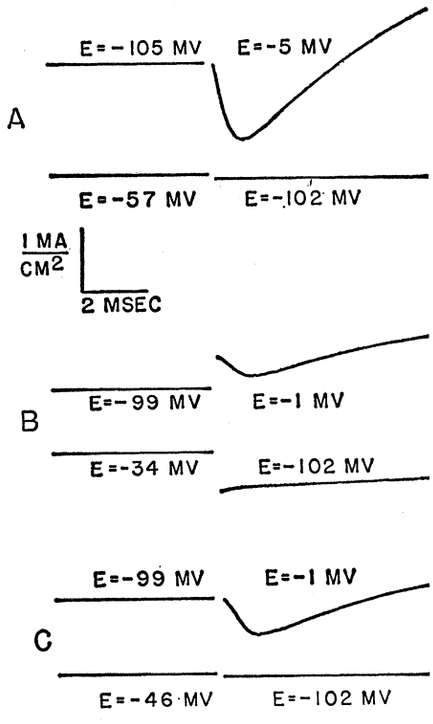

Fig. 1. Comparison of voltage-clamped membrane currents obtained during internal perfusion with potassium chloride with initial and final records with internal perfusion with potassium fluoride. Three sets, *A*, *B*, and *C*, each of two records, are shown. Upper record of each set shows the membrane current flowing during a depolarizing step pulse in membrane potential following a hyperpolarizing prepulse of 50-msec duration; each lower record shows the membrane current flowing during a hyperpolarizing step pulse in membrane potential from the holding potential, which was set equal to the resting potential. Capacitative current transients are not shown. *E*, Clamped potential. *A*, Initial records of membrane current upon internal perfusion with potassium fluoride solution. *B*, Records of membrane current obtained after 30-minute internal perfusion with potassium chloride solution. *C*, Records of membrane current obtained after 15-minute recovery following internal perfusion with potassium fluoride solution. Axon 65–84; temperature, 6°C.

Such current measurements have been used to determine leakage conductance, g_L (*5*). In Fig. 1*B* are two comparable records obtained after 30-minute internal perfusion with phosphate-buffered KCl solution; note that the maximum inward sodium current is greatly reduced; there is also an initial instantaneous step of current not seen before—about 0.4 ma/cm^2. A hyperpolarizing voltage step from a holding potential equal to the resting potential (from −34 to −102 mv) shows a large outward current of about 0.6 ma/cm^2. The instantaneous membrane resistance is only 100 ohm cm^2; initially in KF solution it was about 1000 ohm cm^2, and was as high as 5000 ohm cm^2 in other KF-perfused axons. In KCl solution the decrease in amplitude of the initial sodium component of membrane current was not caused by change in resting sodium inactivation, because the 50-msec prepulse potential of −99 mv was more than sufficient to overcome any resting inactivation.

After 36-minute perfusion with the KCl solution the axon was again perfused with the KF solution. Figure 1*C* shows comparable records of membrane current obtained after 15-minute perfusion with the control solution; while the maximum inward sodium current has not completely regained its initial value, leakage current is essentially the same as it was—representing an instantaneous membrane resistance of about 1000 ohm cm^2.

Figure 2, *A* and *B*, summarizes the results shown in Fig. 1 and compares them with the corresponding record of resting membrane potential (E_{RP}) shown in Fig. 2*C*. Although the resting potential is plotted continuously (Fig. 2*C*), the record was in fact interrupted for brief intervals corresponding to the points in Fig. 2, *A* and *B*, at which the axon was voltage-clamped. In Fig. 2*A* the maximum inward sodium current, I_{Na}, is plotted; in Fig. 2*B*, the leakage current (I_L^*) at − 102 mv. While I_{Na}, I_L^*, and E_{RP} change only slowly during the first 25 minutes of KCl perfusion, change is more rapid after 27 minutes. Within 35 minutes the sodium current drops to zero, leakage currents become very high, and the resting potential becomes low—about −30 mv. These results suggest progressive mild impairment of membrane function with time to a point at which membrane behavior begins to show major alterations.

These results suggest that the decline in action-potential amplitude with chloride perfusion medium parallels

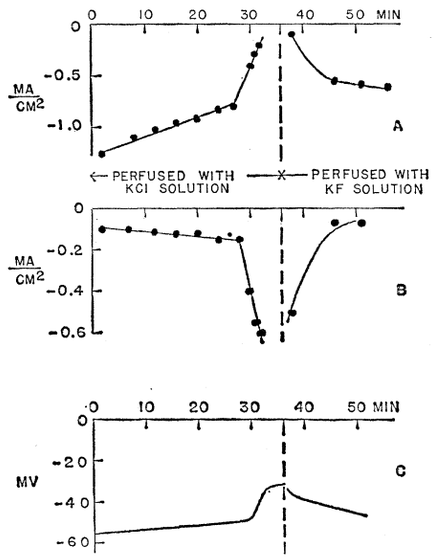

Fig. 2. Alterations in selected membrane currents in the voltage clamp and in membrane resting potential, upon internal perfusion with potassium chloride solution, and their restitution by potassium fluoride. *A*, Maximum initial transient outward (sodium) current values (ma/cm^2), during a brief voltage-clamp period, plotted against the time elapsed in minutes for internal perfusion of axons with potassium chloride solution followed by potassium fluoride solution. Prepulses and depolarizing pulses, similar to those in Fig. 1; all values corrected for leakage currents. *B*, Step outward current (ma/cm^2), upon a change in voltage-clamped membrane potential, from a value equal to the resting potential to a hyperpolarized value of −102 mv plotted against the time elapsed for internal perfusion as in *A*. *C*, Resting-potential values at the times between the points given in *A* and *B*. Axon 65–84; see text and Fig. 1.

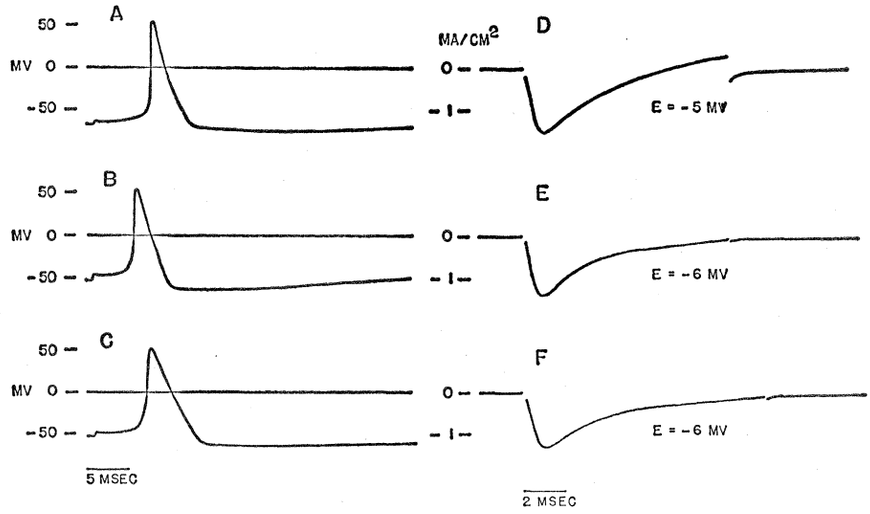

Fig. 3. Demonstration of the protection against the deleterious effects of internal chloride afforded by a low concentration of fluoride in the chloride perfusion medium. Records *A*, *B*, and *C* are membrane action potentials; *D*, *E*, and *F* are maximum sodium currents during a voltage-clamp, depolarizing, step pulse in membrane potential, from the holding potential of —65 mv to the values shown on the records. Records *A* and *D* were obtained initially upon potassium fluoride perfusion; *B* and *E*, after approximately 45-minute internal perfusion with a mixture of nine parts chloride and one part fluoride solution; *C* and *F*, 10 and 15 minutes, respectively, after reperfusion of the axon with fluoride solution. Axon 65–68; temperature, 4°C.

the decline in sodium conductance and coincides with the rise in leakage conductance. This idea suggests that the chloride effect is on some one component of the membrane complex that would alter these values oppositely and exactly in coincidence. Tasaki *et al.* (*1*) have temptingly suggested that the macromolecular structure of the membrane itself is altered by the "unfavorable" anion. The results of less-extensive studies, made with K_2SO_4 internal-perfusion solutions, clearly indicated that $SO_4^=$ was slightly more favorable than Cl^- but considerably less favorable than F^-.

The data in Fig. 3 demonstrate that 90 percent of the favorable fluoride ion may be replaced by the unfavorable chloride anion without the increase in leakage current and decrease in sodium current observed when only chloride ion is present. This fact agrees with the observation of Tasaki *et al.* (*1*) that the ratio of favorable to unfavorable ions must exceed 1 : 10 before the protection of the favorable ion is lost. Explanation of these ion interactions awaits further investigation and understanding of ion-macromolecular interactions in isolated, well-defined systems (*6*).

The initial component of membrane current in the voltage clamp, which has been shown by Hodgkin and Huxley's (*7*) mathematical empirical description of specific membrane conductances to predict the rising phase of the action potential, is greatly decreased by the action of an unfavorable internal anion such as Cl^-. This effect implies that unfavorable internal anions affect the membrane macromolecular conformations in such a way that admission of ions to membranes is altered.

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Enzyme Changes in Neurons and Glia during Barbiturate Sleep

Abstract. *During barbiturate sleep of rabbits, the succinoxidase activity in isolated neurons and glia from the caudal part of the reticular formation was lower than that during physiological sleep. No rhythmical, inverse enzyme changes were detected in barbiturate sleep in the neuron-glia unit, such as were found in physiological sleep.*

In a previous study we have found rhythmic enzyme changes in isolated neurons and glia during sleep and wakefulness (*1*). During sleep, the succinoxidase activity, expressed per nerve cell and unit glia tissue per hour, was increased in the neurons and lowered in the glia in the caudal part of the reticular formation. During wakefulness, this situation was reversed. In the oral part of the reticular formation, rhythmic changes occurred only in the neurons. We also tested the neurons and glia of the hypoglossal and trigeminal mesencephalic nuclei. No changes of the enzyme activity were found during sleep as compared to wakefulness. The observed rhythmic changes were interpreted as indicating that the caudal part of the reticular formation reflects in metabolic changes the biological clock behind the sleep rhythm.

Microchemical studies of the effect of stimulation on neurons and their surrounding glia and the kinetics of enzyme activities have shown that there exists a metabolic coupling between these two types of cells (*2*). The neuron and its glia respond as a unit to functional demands. The oscillation of the enzyme activities in the caudal part of the reticular formation during the circadian sleep rhythm focuses interest on autonomously regulating mechanisms in cells of specific brain regions.

In order to investigate whether sleep induced by drugs is accompanied by similar oscillatory changes in enzyme activities, we have studied neurons and glia in the caudal part of the reticular formation of rabbits after intravenous injection of pentobarbital [0.45 ml/kg; 6-percent solution of 5-ethyl 5(1-methylbutyl) malonylcarbamid, sodium salt, Mebumal, ACO Company, Stockholm]. The animals were killed ½ hour after the injection, by a remote-controlled guillotine. The animals were kept in a soundproof room during the experi-