a hair dryer. Contact of the solutions with the metal of the containers may be avoided by lining the containers with polyethylene bags. This does not affect the freezing rate, provided that the spaces between the bag and container wall are filled with water. The containers must be nearly filled to begin with, but the necessity of having many different sized containers available can be eliminated by using a core frozen to the proper capacity.

Occasionally entrapment will result in losses, and the easiest way to recover the lost material is to thaw the sample and freeze it again. However, any losses which do occur are nonspecific and do not result in fractionation. Thus any easily measured parameter such as conductivity or optical absorbance will serve to indicate the recovery of the whole. Another phenomenon which sometimes occurs is supercooling followed by the formation of ice throughout the solution. It has been found that this rectifies itself and does not interfere with the final result.

Since the success of the procedure, or at least its application to a larger scale than that of Haurowitz, or Gibor, or Schildknecht and Mannl, depends upon stirring, it is worth-while describing what seems to be a plausible hypothesis for its necessity. In a quiescent solution, as ice is formed, the salts eliminated from it dissolve in the adjacent layer of solution and lower the freezing point of this layer. Thus the temperature of the layer is allowed to fall below the freezing point of the rest of the solution. Then, that part of the solution immediately adjacent to the layer, not having the higher salt content of the layer, becomes supercooled and may suddenly freeze, trapping the layer with its content of salts against the ice, where it too subsequently freezes. Thus the purpose of the stirring is to prevent the formation of such layers. This hypothesis of Himes *et al.* (*5*) is supported by the appearance of ice formed during periods when stirring was interrupted. In these experiments such ice differed from the usual clear structureless ice in being laminated in appearance. The laminations, which were several millimeters in thickness, were blue as a result of entrapment of methyl violet which had been added to the solutions (*6*).

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6. In the hope that ready availability of the necessary equipment will encourage use of the methods described, arrangements have been made with the Virtis Company, Inc., Gardiner, N.Y., to manufacture and market such equipment. Part of the work was done during the tenure of U.S. Public Health Service research grant No. RG 7229.

20 February 1961

## Fractionation of Tracer Effluxes during Action Potential

*Abstract.* The fluid flowing by the *Nitella* internode or squid giant axon loaded with radioactive cations was fractionated during the action potential. In this manner it was possible to ascertain the time course of efflux of these cation tracers during activity.

In the investigation with radioisotopes of transport phenomena in biological membranes, it is desirable to correlate the movement of radioisotopes with other rapid processes in the membrane, such as changes in electric potential and membrane conductance. It is known that the process of action potential production in the squid giant axon and in other excitable cells is associated with enhancement of loss of various univalent cationic radio-tracers to the extracellular fluid (for example, *1*). It was difficult, however, to decide with certainty in what phase of bioelectric activity this enhancement occurred. Wilde and co-workers (for example, *2*) have had some success in this direction. By perfusion of the coronary vessel of the whole heart, they were able to obtain a detectable efflux of potassium-42. There are, however, obvious ambiguities in their experiment as to the temporal relationship between the efflux and the electric response of the heart because of the impossibility of ascertaining the time required for diffusion of the isotope. In the present study, it has been possible to fractionate the radioisotope efflux during the action potential in single cells (*Nitella* and squid giant axon).

The principle of the method consists in running the fluid medium past the cell under study at a high speed and then fractionating it. A single cell of *Nitella* or a squid giant axon (both approximately 0.6 mm in diameter and 40 mm long) was introduced into a small chamber (1.2 to 2 mm in diameter) made of Lucite. The cell was kept approximately at the center of the chamber. The wall of the chamber was coated with melted paraffin. There were two pairs of platinum electrodes making contact with the fluid in the cavity. The upper opening of the chamber was connected to a large reservoir of fluid and, by virtue of the difference in the hydrostatic pressure, the fluid in the cavity was pushed through at the rate of about 50 to 150 cm/sec in the case of the *Nitella* cell and about 200 cm/sec in the case of the squid axon.

Fractionation of the running fluid was effected by a rotating Lucite disk (about 70 cm in diameter) provided with 40 to 200 small compartments near its edge. The disk was driven by a motor at about 1 rev in 30 to 60 sec in the case of *Nitella* and at the rate of approximately 1 rev/sec in the case of the squid axon. A source of light attached to the rotating disk and a stationary photocell were used to trigger stimuli (as well as the oscillograph used to record the action potential) at a given position of the disk. The Lucite chamber carrying the cell was held by a movable stand so that it could be quickly brought to, or moved away from, the rotating disk.

The middle diagram in Fig. 1 shows an example of the results obtained with a *Nitella* cell loaded with potassium-42. Electric stimuli were repeated at intervals of 2 min for a total period of 80 min. The running fluid (artificial sea water diluted by 500 with distilled water) was fractionated into portions containing effluxes obtained in 0.75-sec intervals. The samples of fluid in individual compartments of the rotating disk thus contained the radioactive potassium released during 40 successive cycles of activity. These samples were dried and measured by a standard radiation counter.

It is seen in the diagram that there is a small efflux of tagged potassium before stimulation and that this level of efflux was markedly enhanced after the delivery of a brief electric shock to the cell. The action potential recorded by this arrangement is "diphasic." With this action potential regarded as composed of two monophasic action potentials (with one of them reversed in phase and delayed by the interelectrode conduction time), the approximate time course of the potential variation under the upper recording

electrode was reconstructed and shown by the thin continuous line.

In all six experiments on *Nitella megacarpa* and five experiments on different species of *Nitella* (probably *flexilis*), it was found that the peak of the action potential roughly coincides with the peak of the efflux of tagged potassium. The slight discrepancy between the peaks of the two curves can be attributed partly to the difference in the time at which different parts of the cell reach the peak of activity. The retention of the radioactive potassium in some part of the path of running fluid is another factor that is responsible for the discrepancy in the peaks and also for the efflux after the end of the action potential. In a number of more recent experiments, it was possible to obtain a closer parallelism.

In this connection it is to be pointed out that Osterhout (*3*) had predicted an outward movement of $K^+$ during activity in *Nitella*. The time course of the efflux observed can be interpreted as a reflection of the increased membrane conductance during activity (*4*). Results from using cesium-134 and rubidium-86 instead of potassium-42 were qualitatively the same.

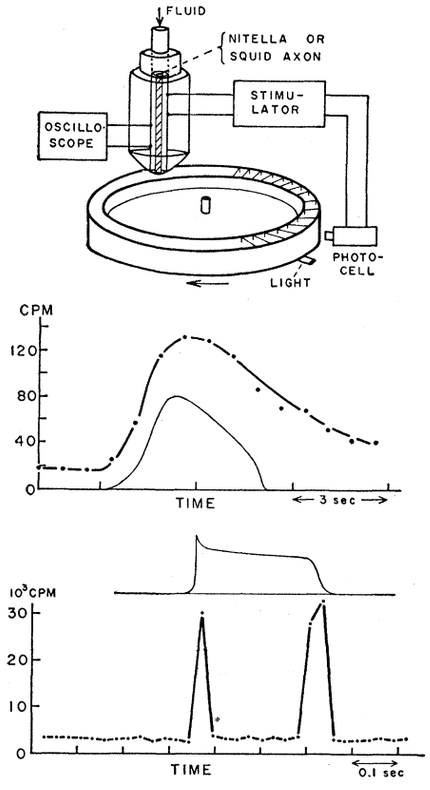


Fig. 1. (Top) Principle of the method of fractionating tracer effluxes during action potential (not to scale). (Middle) Efflux of $K^{42}$ from a cell of *Nitella* (dots) during the action potential (thin continuous curve). (Bottom) Efflux of $K^{42}$ from a squid giant axon treated with tetraethylammonium chloride (dots) during the action potential (thin continuous curve).

Using chloride-36 instead of tagged cations, we tried to demonstrate an increased efflux of tagged univalent anions during activity. We could bring the radioactivity of the cell up to 1000 count/min or slightly more. We could not influence the loss of this radioactivity from the cell by stimulation. We found also that complete substitution of the chloride ion in the medium with glutamate did not bring about any appreciable change in the amplitude of the action potential.

We encountered serious difficulties when attempts were made to apply the same technique to the squid giant axon which requires much higher time resolution in order to correlate the effluxes of tagged ions with the action potential. Such difficulties were partly overcome by prolonging the action potential by injecting tetraethylammonium chloride uniformly over the entire length of the axon. By this procedure, combined with a lowering of the temperature to about 7°C, it was possible to increase the duration of the action potential to 0.5 to 1.0 sec or more. We tried to reduce the thickness of the stagnant layer of the fluid by inducing turbulance in the running fluid around the axon. In several favorable cases, we could attain a time resolution up to the order of 20 msec. In many instances, however, the resolution was poorer than about 100 msec, and, as a consequence, we could not detect an increase in the efflux associated with the action potential. We attribute this variability in the time resolution to the variability in the thickness of the connective tissue layer on the surface of the axon.

The bottom diagram in Fig. 1 shows the result of one of six successful experiments in which the efflux of potassium-42 or sodium-24, or both, was investigated in axons treated with tetraethylammonium chloride. In this example, an isotonic potassium chloride solution containing potassium-42 was injected in a portion of an axon treated uniformly with tetraethylammonium chloride. It is shown in this diagram that there is one phase of increased efflux at the onset and another phase at the end of the prolonged action potential. There was no appreciable increase in the efflux during the plateau. A similar result, with double peaks in the efflux, was obtained with sodium-24. The ratio of the flux at the onset to that at the end varied considerably from preparation to preparation. At this preliminary stage of the experiments, it is not possible to make a definite statement as to this ratio. The results obtained on the relative impermeability of *Nitella* to chlorine-36 are contradictory to the experiments of Gaffey and Mullins on *Chara* (*5–7*).

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6. A report of our interpretation of the experiments presented here and their implications for prevailing concepts of excitation is in preparation. Recently it has come to our attention that C. Terzuolo is in the process of studying the efflux of isotopes from *Nitella* autoradiographically.
7. We are grateful to Mrs. W. J. V. Osterhout for her interest and help.

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## Osteogenic Induction across Millipore Filters in vivo

*Abstract.* Immunization of host mice to homograft tissues prior to subcutaneous implantation of homograft bone within diffusion chambers did not prevent new bone formation on the host side of the filter, thereby indicating its origin from host cells in response to a diffusible osteogenic inductor from within the chamber rather than from undetected escaping homograft cells.

In a previous study (*1*) designed to provide information about the origin of newly formed bone around bone autografts, it was found that subcutaneous implantations of 2- to 3-day-old isologous mouse calvaria within Millipore diffusion chambers (*2*) for periods ranging from 2 to 8 wk resulted in the formation of new bone and cartilage exclusively *within* the chambers. In more recent experiments, bone formation on the host side of the filter (which