6. Food and water consumption were determined empirically by monitoring food and water consumed by six or more lactating females with young. At 22°C and 50 percent relative humidity consumption (grams of Purina rat chow) was 0.21 times the body weight (grams) of the mother plus the young. Consumption (milliliters of water) was 0.29 times the body weight (grams) of the mother plus the young. Food and water rations were always based on the total biomass in the cage.
7. We thank H. W. Norton, W. G. Hunter, P. A. Randolph, and G. E. P. Box for critical reviews. Supported by DOE contract EY-76-S-02-2270.

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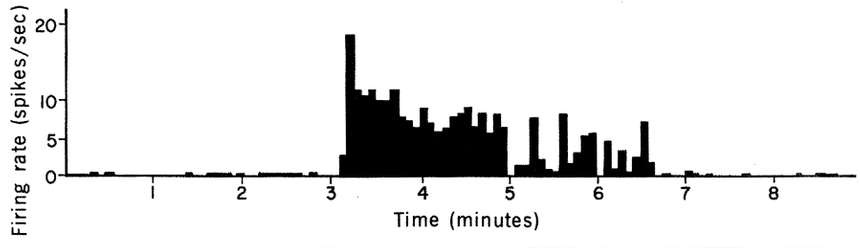
Detection of an Auditory Nerve–Activating Substance

Abstract. *A substance or substances capable of increasing the firing rate of primary auditory fibers is detectable in the perilymph of frogs and guinea pigs subjected to sound stimulation. The increase in firing rate occurs in single units of the frog auditory nerve after perilymph obtained from frogs or guinea pigs during sound stimulation is infused into the frog perilymphatic sac. Perilymph collected from animals maintained in silence failed to cause an increase in firing rate of primary auditory fibers of the frog.*

The primary afferent transmitters of most sensory systems, including audition, remain unidentified. This report presents evidence of the presence of an auditory nerve-activating substance (ANAS) in the perilymph of animals stimulated with sound. Although we are aware of other possibilities, our working hypothesis is that ANAS is the primary afferent transmitter of audition.

Ample evidence indicates that transmission between the labyrinthine hair cells and the auditory (eighth) nerve is chemically mediated. For example, synaptic bodies and synaptic vesicles are present in many cochlear hair cells (*1*), and Furukawa *et al.* have demonstrated the presence of miniature excitatory postsynaptic potentials in the eighth nerve of the goldfish (*2*). The identity of the neurotransmitter released by the hair cells, however, remains unknown. There is evidence that the transmitter is not acetylcholine (*3, 4*), γ-aminobutyric acid (*3, 5-7*), 5-hydroxytryptamine (*8*), glycine (*7*), or a catecholamine (*9, 10*). Although aspartate and glutamate have been suggested as possible candidates (*7, 11, 12*), preliminary evidence from our laboratory is not consistent with that hypothesis (*13*). Transmission across the afferent synapse is resistant to the action of many known pharmacological blocking agents (*3, 5, 9, 12*). Therefore, the transmitter may be a substance not currently listed among the putative transmitters. If it is not, the standard approaches to transmitter identification, such as screening by applying agents from the list of transmitters or standard blocking agents, or the application of radioactively labeled precursors, may not prove fruitful. What is required is a method that will detect the presence of the transmitter regardless of its chemical nature. One answer to this methodological problem is to use the animal as the detector, as Otto Loewi did in his classical experiments with Vagusstoff (*14*). Such a bioassay would permit the detection of the transmitter as well as the monitoring of the progress of efforts to concentrate and to purify it. We have developed such an assay by using the change in firing rate of single nerve fibers of the eighth nerve of the bullfrog (*Rana catesbeiana*) as an indicator of the presence of ANAS in fluid infused into the frog perilymphatic space.



	Withdrawal in silence	Reperfusion in silence	Withdrawal in sound	Reperfusion in silence	Pump off
Mean rate	0.03	0.11	8.1	2.7	0.13
Standard error	±0.01	±0.10	±0.4	±0.5	±0.04

Fig. 1. The results of a typical experiment designed to collect and detect the afferent transmitter. As a control, perfusate was collected in silence. The pumps were reversed, and the collected perfusate was reperfused in silence while the firing rate was being monitored. In order to collect the transmitter, perfusate was collected during sound stimulation. The pumps were reversed, reperfusing the collected perfusate during silence.

The perilymphatic space of the frog was perfused during acoustic stimulation. The collected perfusate was reinfused into the perilymphatic space during silence while we monitored the firing rate of a single auditory fiber of the eighth nerve. As a control, the same unit was monitored during perfusion in silence and infusion of this perfusate into the perilymphatic space during silence. The firing rate during the infusion of perfusate collected in sound was compared to the firing rate during the infusion of perfusate collected in silence. This procedure was based on the assumption that the transmitter, if released into the perilymphatic space during acoustic stimulation, would, when administered during the infusion procedure, produce an increased firing rate when compared to the control procedure.

The frogs were immobilized by the intramuscular injection of 2 to 3 mg of *d*-tubocurarine hydrochloride in 1 ml of saline. The eighth nerve and the perilymphatic sac were exposed through the roof of the mouth. The frogs were subjected to a free-field search stimulus of 60-dB, 300-msec burst of white noise once per second. This stimulus was designed to induce auditory nerve fibers to fire. Single-unit recordings were obtained by using a motorized microdrive to advance a 3*M* KCl-filled glass microelectrode (resistance, 20 to 80 megohms) through the posterior branch of the eighth nerve. Once an auditory fiber was found (as indicated by action potentials elicited in synchrony with the search stimulus), its characteristic frequency was determined by sweeping across frequencies while noting changes in firing rate. The characteristic frequency was used to ascertain its origin in the inner ear (*15*). Only those units originating in the amphibian papilla and exhibiting low spontaneous activity (0 to 5 spikes per second) were used as biodetectors of transmitter-induced activity. The firing rate of the unit during the procedure was recorded on tape and later analyzed with a window discriminator and rate-interval analyzer. The perilymphatic space was perfused with modified Ringer solution (*16*) by means of a double-barreled coaxial pipette inserted into the perilymphatic sac. During the perfusion procedure, perilymph was withdrawn into the inner barrel (diameter, 75 μm). The duration of the perfusion procedure ranged from 60 to 120 seconds. The collected perfusate was then infused back into the perilymphatic space. The rate of perfusion and infusion

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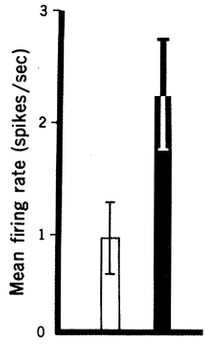

Fig. 2. The mean firing rate in silence of single nerve fibers arising from one of the bullfrog acoustic organs, the amphibian papilla, while perfusate collected during sound stimulation (■) and during silence (□) ($N = 7$) was being infused. The graph summarizes those individual experiments in which the firing rate during the infusion of perfusate collected during acoustic stimulation was significantly ($P < .001$) higher than that during the infusion of perfusate collected in silence. In five experiments performed in the winter months (*20*), there was no significant increase ($P > .05$) in the rate of firing during the infusion of perfusate collected during acoustic stimulation compared with that collected during silence.

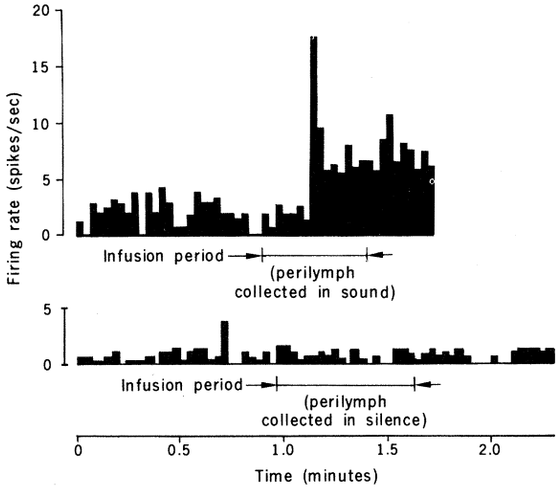

Fig. 3. The firing rate during silence of single nerve fibers arising from one of the bullfrog acoustic organs, the amphibian papilla, before, during, and after the infusion of guinea pig perilymph collected in sound or in silence. The time period between the beginning of the infusion period and the increase in firing rate is accounted for, in part, by a lag time between turning the pump on and the actual movement of perfusate from the pipette (7 to 15 seconds). Similar results were obtained in 11 such experiments. In 17 experiments performed during the winter months (*20*) the infusion of perilymph collected during acoustic stimulation did not result in a significant change ($P > .05$) in the rate of firing of units monitored. In no case did the infusion of perilymph collected during silence ($N = 24$) result in a significant change in the rate of firing of units monitored.

was 4 μl/min. The perfusion procedure was carried out in the presence of pulsed white noise (*17*) or in silence.

The results of a typical experiment are given in Fig. 1. Similar results were obtained in seven such experiments. The infusion of perilymph collected from the frog during acoustic stimulation caused a significant increase in the firing rate when compared with the firing rate during the infusion of perilymph collected in silence (paired t-test, $P < .001$) (Fig. 2). Since the infusion parameters during the control procedure were exactly the same as those during the experimental procedure, the effect could not be due to the infusion process itself. The increase in firing rate was considered to be due to the presence of ANAS released into the perilymph and collected in the perfusate during acoustic stimulation, which, when infused back into the perilymphatic space during silence, stimulated the postsynaptic receptors at the junction of the hair cell and the eighth nerve.

The experimental design called for (i) an initial bioassay for ANAS in a portion of the perilymph followed by (ii) chemical manipulation on the remainder followed by (iii) a second bioassay to monitor the process of concentration and purification. The changes in firing rate induced by the perilymph collected during sound stimulation are relatively small (Fig. 2), indicating that the content of ANAS may be low. The content of ANAS was felt to be too low to carry out the complete experimental design described above. For this reason, a source of greater amounts of ANAS was sought. An attempt was made to collect ANAS released from the mammalian cochlea (guinea pig) containing tens of thousands of sensory cells rather than the hundreds of sensory cells of the frog. Guinea pig perilymph was collected either during acoustic stimulation (*18*) or during silence by a modification of the method of Norris *et al.* (*19*). The samples were infused into the frog perilymphatic space through a single-barreled, siliconized micropipette (tip diameter, 50 μm) placed in the perilymphatic sac. The rate of infusion was 8 μl/min, and the duration ranged from 10 to 30 seconds.

The infusion into the frog perilymphatic space of guinea pig perilymph collected during acoustic stimulation produced a dramatic increase in the firing rate of the single-unit fibers being monitored, compared with the firing rate during the infusion of guinea pig perilymph collected in silence (Fig. 3). The infusion of perilymph collected during acoustic stimulation produced significant increases in the rate of firing in 11 such experiments (t-test, $P < .025$). The mean (± standard error) increase in the rate of firing in these experiments was 3.6 ± 1.9 spikes per second. In no case did the control procedure (the infusion of perilymph collected during silence) result in a significant increase in the rate of firing of monitored nerve fibers. These results indicate that a substance is released into the perilymphatic space during sound stimulation, which, when applied to the perilymphatic space of the frog, induces an increased firing rate in the frog auditory nerve.

We conceive that the primary afferent synapses have some means of terminating the action of the transmitter when the release of that transmitter is within "normal" limits. During intense sound stimulation, those terminating mechanisms may be saturated, and the excess transmitter may diffuse into the perilymph. Diffusion into perilymph may even be a minor terminating mechanism operating normally. Therefore, during intense sound stimulation the perilymph being continuously collected would contain the "overflow" transmitter not otherwise terminated.

Our results suggest that ANAS is detectable in the perilymph of two widely disparate species and that the ANAS crosses species lines in its ability to stimulate receptors. It is as yet too early to know whether ANAS is identical with or related to the primary afferent transmitter of audition.

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16. 115 m*M* NaCl, 1.1 m*M* KCl, 1.2 m*M* $CaCl_2$, 5.5 m*M* glucose, and 2.7 m*M* $NaHCO_3$.
17. The sound stimulus consisted of bursts of white noise of 90 msec duration at ten bursts per second presented in free-field configuration. The white noise intensity measured with the use of octave band filters ranged between 57 dB sound pressure level (SPL) at 125 Hz and 73 dB SPL at 2 kHz. On the C scale of a Bruel and Kjaer sound level meter, an intensity of 78 dB was registered.
18. The sound stimulus consisted of continuous white noise plus clicks presented at seven to ten per second in free-field configuration. The white noise intensity ranged between 45 dB SPL at 125 Hz and 85 dB SPL at 4 kHz. An intensity of 88 dB SPL was registered. The click intensity ranged between 44 dB at 31.5 kHz and 86 dB at 4 kHz. An intensity of 92 dB SPL was registered. This combination of white noise plus click was presented for 5 to 7 minutes with a 5-minute rest period between presentations.
19. C. H. Norris and P. S. Guth, *Acta Otolaryngol.* **77**, 318 (1974). Perilymph was collected by means of a single-barrel micropipette placed in the scala tympani of the basal turn rather than by perfusing the perilymphatic space with artificial perilymph.
20. During the 2-year period of this research, neither guinea perilymph nor bullfrog perfusate, collected during acoustic stimulation, caused an increase in firing rate during the winter months (October through March). This experience recalls the early statement of R. M. Yerkes [*J. Comp. Neurol. Psychol.* **15**, 279 (1905)]: "evidently the seasonal condition of the animal is an important matter to consider in studies of audition."
21. Supported by Veterans Administration grant VA 71-6 (to P.S.G.), Public Health Service grant NS-11647 to the Kresge Hearing Research Laboratory of the South, a John A. Hartford Foundation grant (to C.H.N.), and Public Health Service training grant IT32 G2107172-02 (to W.F.S.). We thank R. R. Capranica for advice and help.

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Use of Indigenous Rubidium to Trace Potassium Fertilizer in the *Pinus resinosa* Ecosystem

The conclusion by Stone and Kszystyniak (*1*) that K was being conserved in the *Pinus resinosa* ecosystem was based upon reduced Rb/K ratios in plants and soil after application of K fertilizer low in Rb. We question the implicit assumption, critical to their conclusion, that the absolute amount of Rb in the soil was not reduced by K fertilization.

In the "reverse tracer" technique of Hafez and Stout (*2*), which Stone and Kszystyniak used, it is assumed that K and Rb remain independent of each other in the soil. However, addition of K may fundamentally change the movement of Rb through the soil-plant system. Fertilization with K may reduce the absolute amount of Rb taken up from the soil by plants as a result of competitive uptake (*3*). This action could also increase the loss of Rb from the ecosystem by leaching, because the sink for Rb in the biomass is reduced by this process. Therefore, the assumption that Rb remains unaffected by K fertilization may not be justified.

Data presented in (*1*) are inadequate to permit one to distinguish between retention of K by ecosystem processes and loss of Rb by leaching. Reduced Rb/K ratios in plant tissues could result from either K retention or Rb loss. The analyses of litter (in milligrams per kilogram of litter) showed reduced concentration of Rb (*1*). In soil (0 to 15 cm deep), the Rb concentration was reduced relative to that of the controls in one stand (Russia, New York) but was essentially constant in the other (North Lawrence, New York) (*1*). Thus soil data showed a possible reduction in Rb concentration in one of the two cases presented. From these data, the loss of Rb after K fertilization cannot be ruled out as a contributing factor in the reduced Rb/K ratios observed.

The reverse tracer technique is potentially useful for investigating the retention of K by ecosystems. However, for valid conclusions to be drawn, the movement of both K and Rb through all parts of the ecosystem, and particularly the interactions between these elements in the soil, must be considered.

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Ryan and Miller raise the possibility that added K may cause leaching of native soil Rb beyond the effective depth of root uptake (*1*) and so account for part of the reduction in plant Rb/K that we reported (*2*). They cite in support the reduced content of 1*N* HNO_3-extractable Rb in the upper 15-cm layer of soil at our Russia, New York, site 23 years after surface application of 112 kg of K per hectare.

There are several objections to this reasoning. (i) Even if the added K had been wholly retained within the upper 15 cm, it would have been equivalent to only about 5 percent of the total exchange capacity, as determined at ambient *p*H. Hence the opportunity for simple mass action displacement of even exchangeable Rb was slight. (ii) Studies of the Rb/K system in mineral soils demonstrate preferential absorption or retention of Rb in both exchange and fixation reactions (*3*). (iii) The samples cited had been subject to the cumulative influence of 23 years of returned litter with a reduced Rb/K ratio, and of canopy wash with presumably a similarly reduced ratio. Studies in a quite different forest show that the exchangeable Ca/Mg ratio of the surface soil approached that of the returned litter despite large point-to-point variations in Ca content (*4*).

The most direct evidence, however, comes from experimental results from a location adjacent to our Russia site. Gritzinger (*5*) measured 1*N* HNO_3-extractable Rb in successive depths of soil 4 months after surface application of 216 kg of K per hectare as K_2SO_4. Mean extractable Rb contents (in milligrams per kilogram of soil for control and fertilized plots, respectively), were as follows: 0 to 10 cm, 0.86 and 0.82; 10 to 20 cm, 0.65 and 0.70; 20 to 30 cm, 0.83 and 0.92; 30 to 40 cm, 0.98 and 1.13; and 40 to 50 cm, 1.11 and 0.93. None of the differences within depth are significant at the 5 percent level. Yet the total precipitation over this period (July to October) was 708 mm, providing opportunity for leaching. During the same period the K concentrations of pine foliage, stems, and roots from treated plots increased and their Rb/K ratios decreased, demonstrating the uptake of applied K. The absence of appreciable displacement of native Rb, even in the surface layer where the added K was initially concentrated, further indicates that the concern expressed by Ryan and Miller is unfounded.

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