The Journal of Neuroscience, August 1987, 7(8): 2465–2475

Neuroactive Substances in Inner Ear Extracts

William F. Sewell[1] and Edmund A. Mroz[2]

Departments of [1,2]Otolaryngology and [2]Physiology, and the [1]Program in Neuroscience, Harvard Medical School, and the [1,2]Eaton Peabody Laboratory of Auditory Physiology, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts 02114

To identify the neurotransmitter released by sensory hair cells, as well as to find other substances that might influence neural function of the inner ear, we have prepared extracts from inner ears of fishes (which have large numbers of hair cells), fractionated the extracts, and studied the effects of the fractionated extracts on the discharge rate of afferent fibers innervating hair cells in the lateral line organ of the African clawed frog *Xenopus laevis*. The extracts contain active substances that do not bind to a cation-exchange resin at neutral pH. Gel-permeation chromatography suggests that at least 2 unidentified excitatory substances are present in the extracts: one of low molecular weight (M_r about 200) and one of high molecular weight (M_r less than or equal to 5000). Some extracts also contain a high-molecular-weight inhibitory substance (M_r greater than 5000). The low-molecular-weight active substance is detected in extracts of inner ear, but not in brain or muscle. The high-molecular-weight excitatory substance is present both in brain and in inner ear.

The neurotransmitter released by hair cells, the sensory cells of the inner ear, has not yet been identified. Over the years, many neurotransmitter candidates have been suggested as being the hair-cell neurotransmitter, including acetylcholine (Gisselsson, 1950; Vinnikov and Titova, 1964), GABA (Flock and Lam, 1974), catecholamines (Osborne and Thornhill, 1972), and glutamate or aspartate (Bobbin and Thompson, 1978). None of these substances has been proven by subsequent work to be the neurotransmitter (Guth et al., 1981); it is possible that the neurotransmitter is a substance not previously suspected to be a neurotransmitter.

If the hair-cell neurotransmitter is not one of the known putative neurotransmitters, trying to identify it through pharmacological and neurochemical screening of neurotransmitter candidates will not be fruitful. An approach that can succeed in identifying this neurotransmitter, even if the neurotransmitter is an unsuspected candidate, is to isolate substances that have neurotransmitter-like activity from tissue that contains hair cells. This approach can potentially lead to the identification not only of the hair-cell neurotransmitter but also of any other substance present in the inner ear (such as excitatory or inhibitory neurotransmitters in the efferent nerve endings) that can influence activity in the afferent nerve fibers. Identification of these substances would then allow detailed study of their effects on inner ear function.

In the initial stage of this approach, Sewell et al. (1978) have demonstrated that some substance capable of activating auditory nerve fibers is released in greater quantities during acoustic stimulation than during silence. This work, however, does not provide enough neuroactive material to allow characterization of the active substance.

We have made 2 significant modifications to this initial approach that greatly increase its utility. First, in order to increase the amount of neuroactive substances available as starting material for study, we have studied extracts of inner ears rather than the perfusates of perilymphatic space, which were studied by Sewell et al. (1978). We have used fish inner ears as sources of tissue for these extracts. In comparison to mammalian ears, fish inner ears are easily accessible, yet contain large number of hair cells. Second, to allow routine detection of neuroactive substances in these extracts, we have developed a bioassay based on a sensory organ with a hair cell–afferent fiber synapse similar to that in inner ears, the lateral line organ of *Xenopus laevis*. Unlike the earlier bioassay (Sewell et al., 1978), which required micropipettes to monitor afferent nerve discharge rates, the *Xenopus* bioassay involves use of a wire electrode, so that activity from the same set of fibers can be recorded for several hours. Thus, the dozens of samples generated during chemical fractionation studies can be bioassayed conveniently, with all the samples from a fractionation step often being tested on the same piece of tissue. We report here that substances extracted from the inner ears of fish can excite afferent fibers innervating the hair cells of the lateral line organ of *Xenopus laevis*, and describe the results of initial separations and the characterization of these compounds.

Materials and Methods

Bioassay

Animals. Immediately postmetamorphic *Xenopus laevis* (African clawed frogs), approximately 2 cm from nose to vent, were obtained from Nasco (Fort Atkinson, WI) and housed at room temperature in deionized water containing 1 mM added calcium chloride.

The lateral line organ of *Xenopus laevis* consists of a series of "stitches" distributed on the head and body of the animal. Each stitch contains 3–10 neuromasts; each neuromast has 10–30 hair cells. Each stitch is innervated by 2 large myelinated afferent fibers, one myelinated efferent fiber, and several unmyelinated fibers (Harris and Milne, 1966).

Recording afferent nerve activity. Each frog was anesthetized by chill-

Received October 13, 1986; revised February 6, 1987; accepted February 9, 1987.

We wish to thank the staff of the Eaton Peabody Laboratory, especially H. M. Gilligan, A. Mendez, and F. Stefanov-Wagner. We thank J. Corwin and A. Popper for their suggestions. We appreciate the trout donated by the U.S. Department of Wildlife and Fisheries and thank R. Colantuno and the staff of Pocono Springs in Myerstown, Pennsylvania, for supplying trout inner ears. We thank G. Adams and F. Mararchi of Scituate Harbor, Massachusetts, for supplying skates. It is a pleasure to acknowledge the contributions of N. Y. S. Kiang. We are grateful to J. Irwin and H. Schuknecht for their support. This research was supported by the NIH Grants NS 19097 and NS 13126.

Correspondence should be addressed to William F. Sewell or Edmund A. Mroz, Eaton-Peabody Laboratory, Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114.

ing to near 0°C, then decapitated. A piece of skin containing the middle-lateral row of stitches was removed and placed, inner side up, on a piece of moistened filter paper. The skin was rinsed with a perfusion solution containing sodium chloride (120 mM), potassium chloride (3.5 mM), calcium chloride (1.5 mM), and glucose (5.5 mM), buffered with HEPES (20 mM), and adjusted to pH 7.5 with sodium hydroxide (total Na^+, 130 mM). Perfusion of the inner surface of the skin allowed rapid diffusion to the basolateral surface of the sensory epithelium.

The nerve branch innervating the middle-lateral row of stitches was dissected from the inner surface of the skin. Nerve activity was recorded by pulling the nerve into a polyethylene tube attached to a 1 ml syringe into which a silver–silver chloride wire had been placed, and then lifting the tube out of the fluid that bathed the skin. The electrical signal was amplified 1000-fold and monitored on an oscilloscope. Monophasic action potentials with positive polarity were observed. The signal-to-noise ratio was optimized by filtering the signal; in general, this ratio was over 10. In most cases, the activity of 3 or 4 stitches (6–8 fibers) was monitored. (With more than 3 or 4 stitches, it became difficult to distinguish individual action potentials in the electrical signal.) Other stitches with afferents running in the nerve branch were crushed.

A ring, 2 mm i.d. and 0.5 mm high, was cut from Silastic medical grade tubing (Dow Corning, Midland, MI) and placed over the monitored stitches; the ring was continually perfused with the solution described above. In early experiments, the perfusion rate was 2 μl sec^{-1}; in later experiments, the rate was lowered to 1 μl sec^{-1}, which allowed application of smaller sample volumes. Excess fluid flowed out of the ring to a drain via a paper wick. The preparation was kept inside a Faraday cage.

Applying samples. Samples with volumes of either 300 μl (when the flow rate was 2 μl sec^{-1}) or 100 μl (when the flow rate was 1 μl sec^{-1}) were administered by diverting the flow of perfusion fluid through a loop of tubing that contained the sample. Since the pump and sample loop were located outside of the Faraday cage, and since turning the valve to apply the sample interfered little with fluid flow, there was no change in discharge rate due to gross mechanical disturbance of the bioassay preparation during application of the sample.

Data analysis. A Schmidt trigger device was used to signal spike occurrences in the afferent fibers of the monitored stitches.The output of the Schmidt trigger device entered a PDP 11/34 computer, which stored the total number of spikes per sec. Firing rates were expressed as the total discharge rate per sec of all fibers monitored in the preparation. The effect of a sample was calculated as the percentage change of the average firing rate from a control period to a period during the maximum effect of applied samples. The precise choice of the control and sample periods depended on the fluid flow rate, the volume of the sample, and the volume of the tubing from the sample loop to the outlet of the perfusion tubing, which were varied as these methods were developed. Control and sample periods were thus chosen empirically, on the basis of the time course of the effects of applied samples (see Results). Once control and sample periods were chosen for a given combination of flow rate, sample volume, and tubing volume, however, they were used for all samples studied under that set of conditions. The periods chosen were consistent with the time course of the appearance of dyes at the outlet of the perfusion tubing. In our current preparation, with which most of the data were obtained (1 μl sec^{-1} perfusion rate and 100 μl samples), we used a period of 70–140 sec after turning the injection valve as the period of the sample's maximum effect, and a period from 50 sec before to 50 sec after turning the injection valve as the control.

Bioassay of samples. Samples to be applied to the bioassay were adjusted so that the pH, calcium, sodium, and potassium concentrations were those of the perfusion medium. In general, each sample was applied to the bioassay 3 times on 3 separate preparations. (In some early experiments with tissue extracts, when sample volumes of 300 μl were used and the volume of extracts was limited, samples were applied to the bioassay twice.) The percentage changes in discharge rate due to the applications of each sample were averaged.

Tissue sources

Goldfish (*Carasius auratus,* "comet" variety), 5–7 cm in length from the nose to the base of the caudal fin, were obtained from local suppliers. Brook trout (*Salvelinus fontinalis*) 25–30 cm long were obtained live from a local hatchery. Skates (*Raja ocelata* and *Raja clavata*), 30–100 cm in length from snout to tip of tail, were obtained from local fishermen.

Goldfish and trout were decapitated, the cranial cavity was exposed dorsally, and the brain lifted up out of the cavity. The otolithic organs were removed by grasping the VIIIth nerve and saccular otolith with a pair of forceps. This procedure removed not only the saccule and its otolith but also the nerve and macula of the lagena. The utricle was then removed from the cranial wall, usually along with the ampullas of the semicircular canals. Visualization through a dissecting microscope at 16× was necessary to remove the inner ears from the goldfish.

Skates were pithed, and the portion of cranium containing the inner ears was removed from the animal. The lateral edges of this piece of tissue were pared down with a knife until the saccules were visible. The saccules were then scraped into a tube.

For most experiments the inner ears obtained from these fishes were subjected to one of 2 general treatments. With one method, the ears were dropped into boiling water, boiled for at least 20 min, and homogenized. With the second method, they were dropped into a saline solution similar to the perfusion medium of the bioassay but containing 30 mM (goldfish and trout) or 150–200 mM (skate) potassium chloride. The ears were then incubated in the high-potassium solution for 45 min. The homogenate or high-potassium bath was centrifuged and the supernate saved for study.

In some pilot studies, inner ears were placed instead in 2 M formic acid or 2 M ammonia, homogenized, centrifuged, and the supernates saved for study. Since acid dissolved the otoconia, the formic acid extracts were further passed through a column of Chelex cation-exchange resin (Bio-Rad Industries, Richmond, CA) to replace the calcium in the extracts with sodium.

For control studies, pieces of goldfish muscle, brain, or eye of mass similar to that of the goldfish inner ears used were dropped into tubes containing the 30 mM potassium saline solution and incubated for 45 min. The tubes were then centrifuged and the supernates removed for study. Goldfish brains were also dropped into boiling water, homogenized, and centrifuged. The supernate was then removed for study.

Chromatographic methods

The crude supernates obtained from goldfish (which provided most of our data) were lyophilized and resuspended in water to yield the equivalent of the ears of 100–200 fish/ml. Supernates from trout were concentrated to the equivalent of about 50 fish/ml; supernates from skates to about 5 fish/ml. When necessary, the concentrated supernates were clarified by centrifugation.

The concentrated supernates obtained from tissue were passed through a column of cation-exchange resin (AG50W-X12; Bio-Rad) in the sodium form to exchange K^+, Mg^{2+}, Ca^{2+}, and other cations, which might affect the bioassay, for Na^+, which could be determined in chromatography fractions by flame photometry and then adjusted to the concentration in the bioassay perfusion fluid. The volume of resin was chosen to ensure at least a 5-fold excess of equivalents of exchange sites on the resin (nominally 2.3 mEq/ml) over the total cation equivalents in the processed supernate. In most cases, where 1–10 ml of concentrated supernate was processed, a 1 or 2 ml column of cation-exchange resin sufficed.

Gel-permeation chromatography was performed with Sephadex G10 and G25 (Pharmacia). The elution buffer contained 20 mM NaCl and 10 mM HEPES, adjusted to pH 7.5 with NaOH (total Na^+, 25 mM). With this buffer, most samples eluting from the columns could be concentrated 2-fold for bioassay without raising the concentrations of these constituents over those of the bioassay perfusion fluid. Column sizes were chosen on the basis of the amount of tissue being processed, so that peak fractions after chromatography would readily show activity in the bioassay. In most cases, the column sizes were 0.9 cm in diameter × 50 cm long, or 2.5 cm in diameter × 50 cm long. Columns were run at linear flow rates between 2 and 6 cm/hr.

Chemical assays

The following assays were adapted to sample volumes of 5–10 μl and performed on fractions from chromatography: protein by Coomassie blue binding (Bradford, 1976); ATP, glucose, glutamate, aspartate, asparagine, total carbon dioxide, and GABA by NAD(P)H-coupled assays performed spectrophotometrically or fluorometrically (Lowry and Passonneau, 1972; Bergmeyer, 1974); primary amines by ninhydrin; HEPES (after separation from protein by gel-permeation chromatography) by the protein assay of Lowry et al. (1951; cf. Good and Izawa, 1972); inorganic phosphate by phosphomolybdate (Cooper, 1977); chloride by titration against mercuric nitrate (Sigma procedure no. 830); ammonium

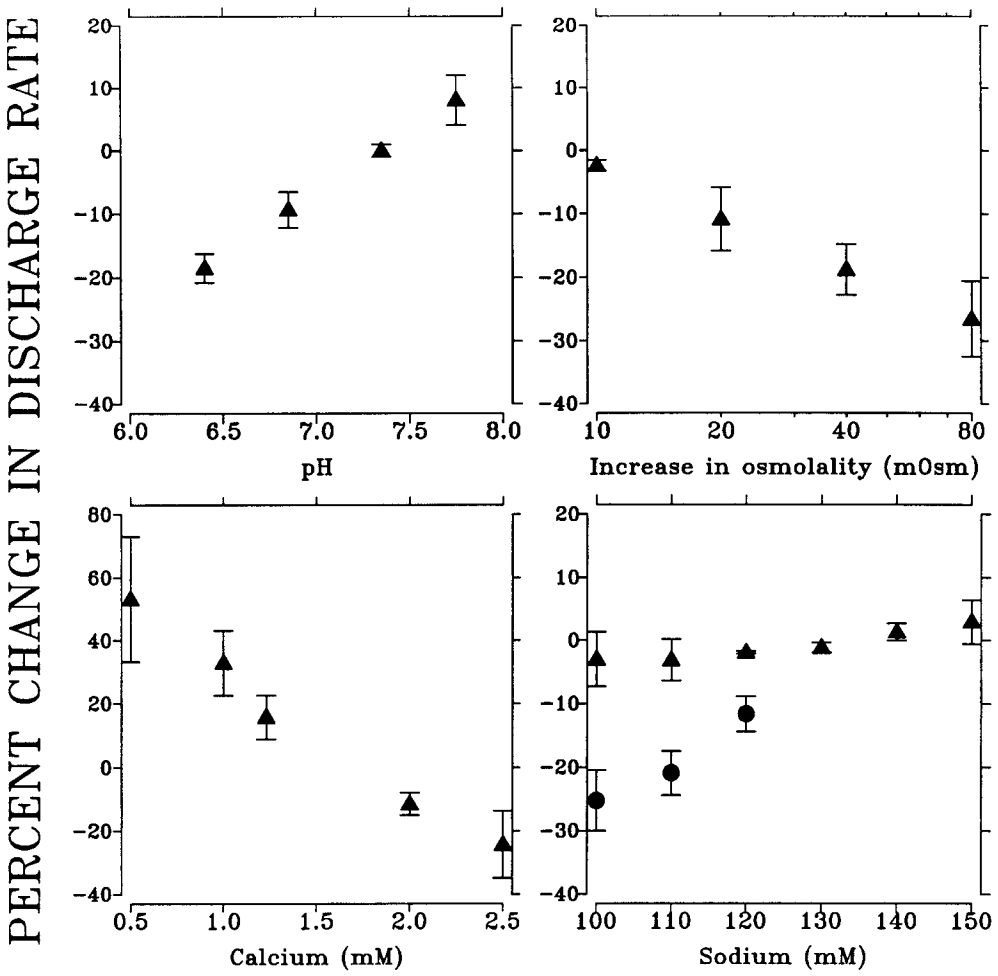

Figure 1. Effects of the composition of applied samples on discharge rates of the bioassay. The mean change in discharge rates of the bioassay (±SEM, 4–11 samples/point) is plotted as a function of the pH, osmolality, and sodium and calcium concentrations of samples otherwise identical with the standard perfusion fluid. Osmolality was increased by adding sucrose. Increases in sodium (over 130 mM) produced an increased osmolality that was not compensated for. Decreases in sodium concentration were either compensated for the decrease in osmolality by addition of sucrose (*circles*) or were not (*triangles*).

with *o*-phthalaldehyde and thioglycolic acid (Mroz et al., 1982); urea and glutamine by that ammonium assay after enzymic treatment to release ammonium (cf. Bergmeyer, 1974); sodium, potassium, and total calcium by flame photometry. Protein, glutamate, glucose, and sodium were monitored routinely to characterize the separations effected by each chromatography column and to allow comparisons among columns. The other substances were measured as required to ensure proper adjustment of samples for bioassay and to determine whether known substances might be present at high enough concentrations to account for the bioactivity we found. The pH of samples to be bioassayed was determined with a micro-combination pH probe (model MI-410, Microelectrodes, Londonderry, NH). In some samples prepared for bioassay, calcium activity was measured with a calcium-selective electrode (CAL-1; World Precision Instruments, New Haven, CT).

Chemicals used in these studies were of reagent grade and were obtained from commercial suppliers.

Results

Spontaneous discharge rates in the lateral line organ changed with pH, osmolality, and ionic composition of the perfusion medium

As reported by others (Harris and Milne, 1966; Zimmerman, 1979), afferent fibers innervating hair cells in the *Xenopus* lateral line organ produced action potentials in the absence of applied mechanical or chemical stimuli. The overall control discharge rate of the 6–8 fibers monitored in a preparation was generally between 20 and 120 sec^{-1}. Since the preparation we used was not completely isolated from environmental vibrations and was continually perfused with fluid, we could not determine whether the activity we recorded was true "spontaneous" activity or the response of these fibers to environmental stimuli.

Since initial studies indicated that the effects of active samples were likely to be best expressed as percentage changes in the discharge rate, we chose experimental conditions that led to low spontaneous percentage changes in this rate. We found that the coefficient of variation of the discharge rate was consistently lower when afferents from more than one stitch were monitored. This was expected from the results of previous studies (Harris and Milne, 1966), which showed a predominantly Poisson-like distribution of single fiber discharge rates. For example, in one preparation in which we systematically lowered the number of stitches kept intact, the overall discharge rate per second from 3 stitches showed a 20% coefficient of variation (over 1000 sec: mean, 19.4 sec^{-1}, SD = 4.1), while the same preparation with one stitch intact showed a 40% coefficient of variation (over 1000 sec: mean, 7.7 sec^{-1}, SD = 3.1). Thus we chose to record from 3 or 4 stitches in most cases.

The spontaneous discharge rate of these fibers varied with the pH and ionic content of the perfusion medium. In our early preparations, we used a perfusion fluid with a higher calcium concentration (3 versus 1.5 mM) and lower sodium concentration (120 versus 130 mM) than in the perfusion fluid described in Materials and Methods. With this earlier perfusion fluid, we found that the discharge rate of the bioassay was low and declined to near zero over a few hours. With immediately postmetamorphic frogs and the perfusion fluid described in Materials and Methods, changes in the control discharge rate were

slow in comparison with the effects of applied samples, and samples could generally be tested for 4–5 hr.

Because our method of sample application required complete replacement of the perfusion medium with sample, it was important to quantify the effects of alteration in the osmolality, pH, and ionic content of the sample on the discharge rate of the bioassay. As shown in Figure 1, increases in osmolality (with added sucrose) and changes in pH and calcium in samples could affect the bioassay. Lowering the NaCl while maintaining osmolality with sucrose led to decreases in discharge rate. If osmolality was not maintained when NaCl was lowered, there was little effect on the bioassay; presumably, the inhibition due to lowered NaCl was offset by an excitation due to the lowered osmolality. In addition, elevations in magnesium concentration depressed the discharge rate of the bioassay, and elevations in potassium produced a mixed excitation–inhibition (data not shown).

The pH of the extracts could be controlled by buffering with HEPES and titrating; the osmolality could be controlled by diluting the extracts until they were isosmotic with the perfusion medium. Tissue extracts, however, also contained potassium, calcium, and magnesium, all of which affected the bioassay. Thus, to control the content of these ions in the bioassay, we treated the extracts with a cation-exchange resin. Since the bioassay was least sensitive to changes in sodium, we used the AG50W-X12 cation-exchange resin column in the sodium form. We found that more than 99% of the potassium and calcium was removed from tissue extracts by passing them through this resin. Potassium chloride and calcium chloride could then be added back in known amounts, so that their concentrations were the same as those of the bioassay perfusion fluid. This treatment with ion-exchange resin also made it feasible to try to obtain active substances by exposing the hair-cell tissue to depolarizing concentrations of K^+ and then removing the K^+ with the cation-exchange resin.

Application of cobaltous chloride rapidly and reversibly reduced spontaneous discharge rate

We performed control experiments to determine whether substances applied to the inner surface of the skin reached the hair-cell-afferent nerve synapse. In these experiments, we replaced calcium in the sample with varying amounts of $CoCl_2$, which competitively blocks calcium-dependent neurotransmitter release (Weakly, 1973). Since activity in lateral line afferents is thought to require transmitter release from the hair cells (Harris and Flock, 1967; Flock and Russell, 1976), application of $CoCl_2$ should decrease the discharge rate of nerve fibers over a time course consistent with its arrival at the synapse. Cobalt chloride, in concentrations similar to those used by Weakly (1973) at the neuromuscular junction, did reversibly reduce the discharge rate of the lateral line afferent fibers within a few seconds of its appearance at the outlet of the perfusion tubing over the inner surface of the skin (Fig. 2). This result suggested that applied samples reached the synaptic area rapidly. The effect of cobalt on discharge rate is unlikely to be due to an effect on transduction, since cobalt has been shown to have negligible effects on transduction in the lateral line organ at the concentration of calcium used in this study (Sand, 1975). Responses to $CoCl_2$ were much more delayed (up to 45 sec) when larger frogs (4–5 cm from tip of nose to vent) with thicker skin (300 μm versus less than 100 μm) were used. Thus we used immediately post-metamorphic frogs for the bioassay.

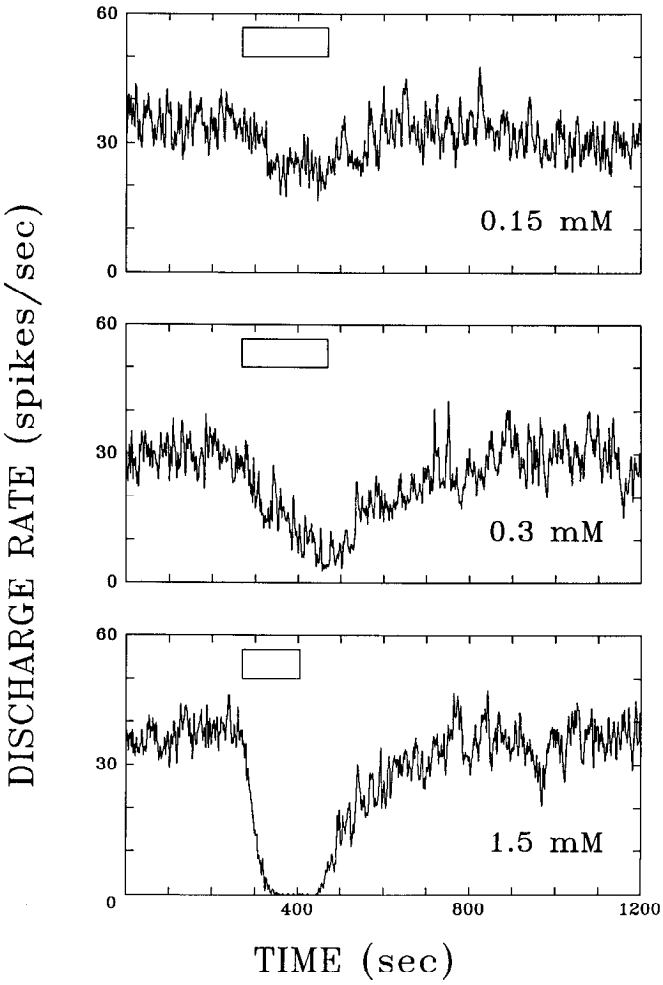

Figure 2. Effects of cobalt on the discharge rate of the bioassay. The discharge rates of fibers innervating hair cells of the lateral line organ in *Xenopus laevis* are plotted as a function of time. Samples in which $CoCl_2$ at the indicated concentrations was substituted for calcium were applied to the bioassay. *Open boxes* indicate the time of delivery to the preparation of the samples, as determined colorimetrically with dyes injected with this delivery system. The same bioassay preparation was used for all 3 applications.

Inner ear extracts excited the bioassay

Extracts obtained by treating inner ears with depolarizing concentrations of potassium, followed by cation-exchange treatment and adjustment of the ionic composition to that of the bioassay perfusion fluid, activated the bioassay (Fig. 3 and left panel of Fig. 4). Passing the high-potassium salt solution alone through the cation-exchange resin and adjusting the effluent from the resin for bioassay did not produce active samples, which ruled out a nonspecific effect of the ion-exchange treatment step. Furthermore, extracts obtained by treating similar amounts of brain, muscle, and whole eye in this way did not activate the bioassay. These results suggested that inner ears do contain substances active on afferent fibers innervating hair cells, and that the concentrations of these substances in the inner ear may be higher than the concentrations in the other tissues we examined.

Treatment with cation-exchange resin served as a preliminary separation step in isolating active substances, since any small substance that is a cation at neutral pH should have bound to

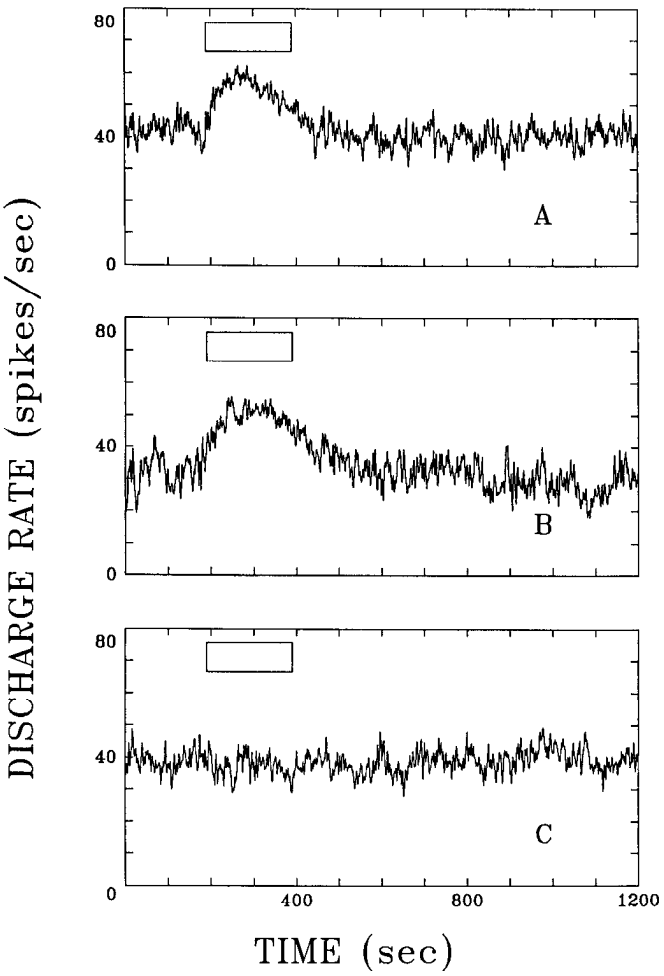

Figure 3. Effects of goldfish inner ear extracts on the bioassay. The discharge rates of fibers innervating hair cells of the lateral line organ in *Xenopus laevis* are plotted as a function of time. *Open boxes* indicate the time of delivery to the preparation of the extracts, as determined colorimetrically with dyes injected with this delivery system. *A, B,* The response of two different bioassays to the same inner ear extract obtained by treatment with depolarizing concentrations (30 mM) of potassium followed by treatment with a cation-exchange resin and adjustment of ionic composition and pH to that of the perfusion medium. *C,* The effect of a control solution (30 mM KCl, 120 mM NaCl, 1.5 mM $CaCl_2$, 20 mM HEPES, 10 mM NaOH, 5.5 mM glucose) subjected to cation-exchange resin, adjusted for ionic composition and pH and applied to the bioassay.

the column and not have appeared in the eluate, as we verified for potassium and calcium.

Gel-permeation chromatography of inner ear extracts separated at least 2 excitatory substances

The extracts described to this point contained many substances, so it is possible that the activity of inner ear extracts was due to more than one substance, or that the lack of activity of extracts of other tissues reflected a combination of excitatory and inhibitory effects. To begin isolating the active substances in the extracts, and to determine whether multiple active substances were present in them, we next fractionated these extracts by gel-permeation chromatography on Sephadex G10, a gel-permeation medium with a nominal high-molecular-weight cutoff of 700 for globular substances. We expected that the presence of an excitatory substance in the extract would result in a peak in a plot of biological activity versus elution volume in the effluent, and that the width of the peak of biological activity in such a plot would be similar to the width of chemically determined peaks for known substances eluting from the column.

Chromatography of inner ear extracts on Sephadex G10. Figure 4 (right panel) is an averaged profile of the changes in firing rate of the bioassay due to application of fractions obtained from G10 chromatography of extracts of goldfish and trout inner ears. Two peaks of excitatory activity were seen, one eluting with the protein and one eluting approximately with glucose. As expected, the widths of these peaks of excitatory activity were similar to the widths of peaks of chemically determined substances (protein and glucose) eluting from these columns.

The chromatogram presented in Figure 4 shows an averaged profile from 5 columns. We have performed additional separations of 11 more concentrated inner ear extracts with Sephadex G10 chromatography. Five extracts (3 goldfish and 2 trout) were obtained by high-potassium treatment. Six extracts (all goldfish) were obtained with boiling water. A low-molecular-weight peak of activity was seen in chromatograms of all 11 extracts. A high-molecular-weight peak was seen in chromatograms of all of the extracts obtained by treatment with high potassium, but in only 2 of those obtained with boiling water. A low-molecular-weight peak was also seen in both chromatograms of skate extracts (high-potassium treatment), in which the equivalent of the inner ears of more than 3 skates/ml was applied to the bioassay.

If these peaks of biological activity were due to substances eluting according to molecular size (the primary determinant of behavior of substances in gel-permeation chromatography), the first would be due to a substance or substances with M_r greater than 700 and the second to a substance or substances with M_r approximately 200. For simplicity, we refer below to these peaks of excitatory activity as the "high-molecular-weight excitatory peak" and the "low-molecular-weight excitatory peak," although factors other than molecular weight (e.g., molecular shape, aromaticity, and charge) can determine the elution of substances from Sephadex G10 columns.

This chromatographic step separated both these peaks of activity from aspartate and glutamate (Fig. 4), 2 amino acids known to affect the firing rates of *Xenopus* lateral line afferent fibers when applied in millimolar concentrations (Bobbin et al., 1981). In the fractionations summarized in Figure 4, the concentrations of these amino acids were too low to substantially affect the firing rates of the bioassays.

Chromatography of extracts from other tissues. Extracts from goldfish brain (boiling water treatment) and goldfish muscle (potassium treatment) were applied to Sephadex G10, and the fractions adjusted and bioassayed in the same manner as were inner ear extracts. The low-molecular-weight peak of activity was not detected either in brain or muscle extracts. A high-molecular-weight excitatory peak was detected only in highly concentrated extracts of brain tissue, when more than 0.5 gm of starting tissue/ml of sample was applied to the column. Both brain and muscle contained glutamate and aspartate; in the fractions containing peak concentrations of glutamate and aspartate, these amino acids were in a concentration sufficient to affect the bioassay.

The low-molecular-weight excitatory peak was related to the number of hair cells processed and applied to the bioassay

We examined whether the amount of excitation of the bioassay caused by these low-molecular-weight fractions was related to

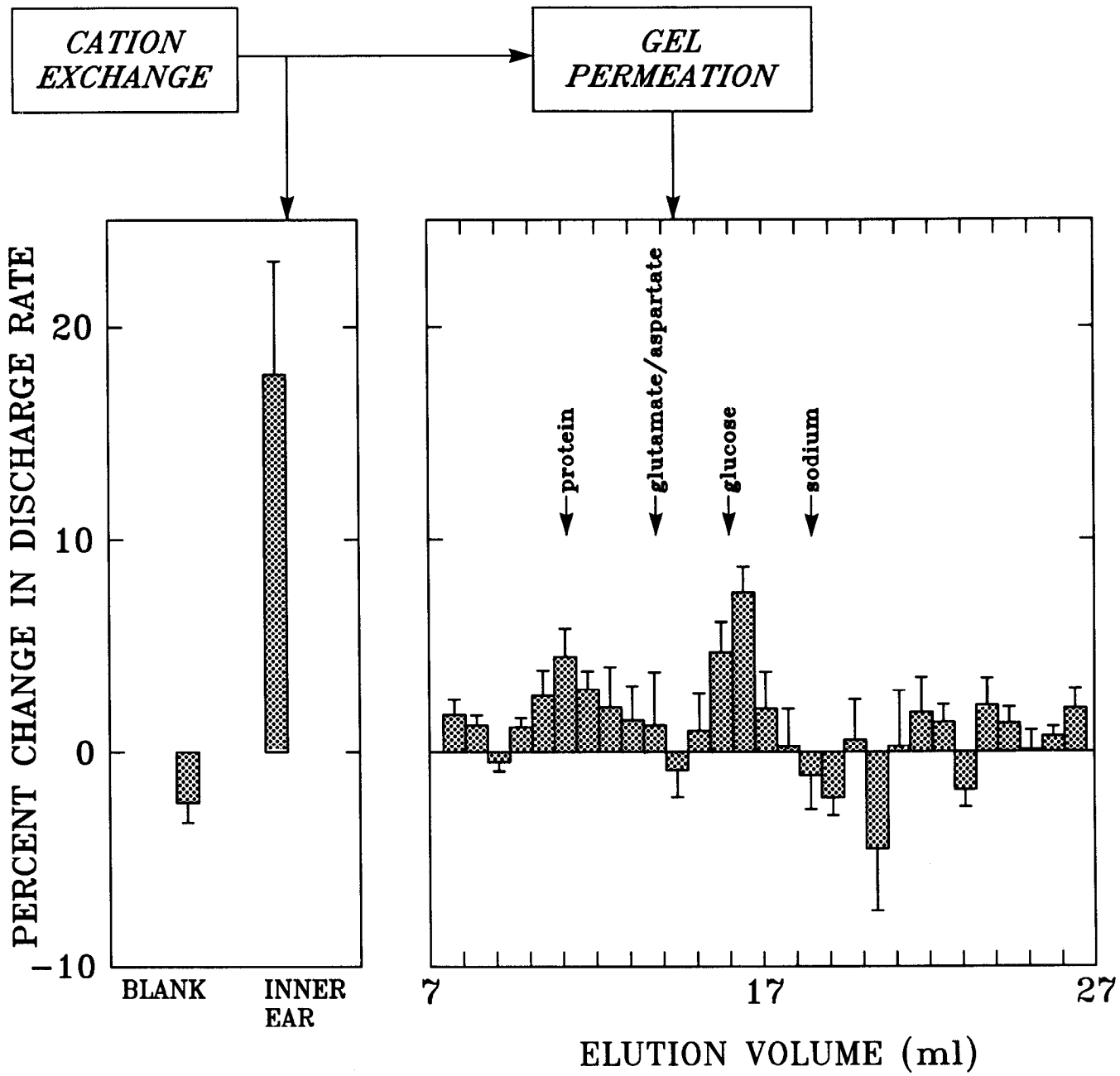

Figure 4. Effects of fractionated extracts of goldfish inner ears on the bioassay. In both panels, *vertical bars* represent the mean (±SEM) changes in firing rate of the bioassay during application of extracts or fractionated extracts, adjusted to the ionic composition and pH of the bioassay perfusion solution. *Left, Inner ear* is the response of the bioassay to 5 extracts, each tested in duplicate after they were passed through a cation-exchange resin, as with the records shown in Figure 3. *Blank* is the response to salt solutions subjected to the same treatment (see legend, Fig. 3). *Right,* The response of the bioassay to fractions obtained from gel-permeation chromatography of 5 preparations of hair-cell tissue, each chromatographed separately on Sephadex G10 after cation-exchange treatment. The *bars* represent the averaged effects of fractions eluting at the indicated volumes from the G10 columns. Given the size of the column (0.9 cm diameter × 50 cm long), the sizes of the samples applied (1–1.5 ml), and the size of the fractions taken (0.75 ml), any given substance should have appeared in 2 or 3 successive fractions. The elution volumes of protein (not retained by this column), glutamate, glucose, and sodium are indicated by the *arrows*. The 2 peaks of excitatory activity that elute near the protein and near glucose correspond, respectively, to what we have termed the high- and low-molecular-weight excitatory peaks.

the number of hair cells processed and applied to the bioassay. Figure 5 is a plot of the percentage change in the discharge rate of the bioassay versus the estimated concentration of extracted hair cells applied to the bioassay for low-molecular-weight peak fractions. This plot suggests that the biological activity of this peak was proportional to the number of hair cells applied to the bioassay. The points for the 2 trout extracts fell within the range of the goldfish extracts, consistent with the presence of the active substance(s) in hair cells of both trout and goldfish. We could not obtain sufficient quantitative data for the number of hair cells in skate saccules to allow us to examine whether the low-molecular-weight peak from skates also fell within this range.

Characteristics of the low-molecular-weight peak

Extracts of goldfish ears were prepared both with high-potassium treatment and with boiling water; there was no systematic difference in the biological activity of the low-molecular-weight

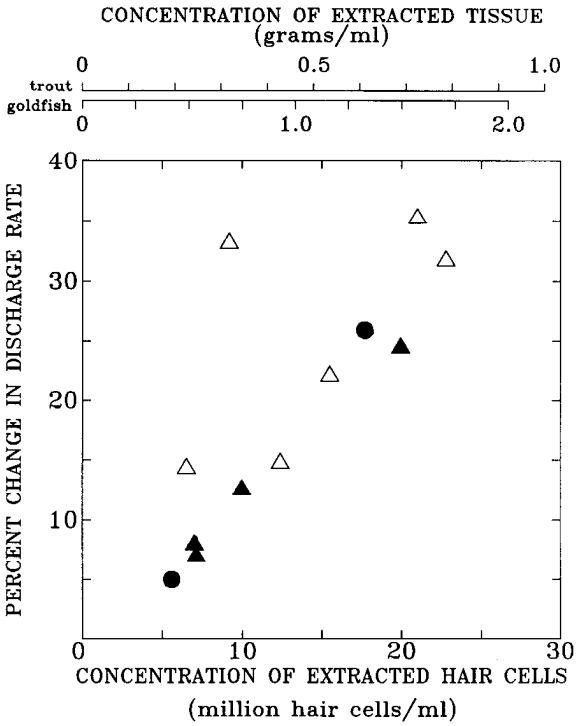

Figure 5. Relation of low-molecular-weight activity to estimated concentration of extracted hair cells and the relative tissue concentration applied to the bioassay. The mean percentage change in discharge rate of the low-molecular-weight peak was determined for each of 11 columns. Data obtained by treatment with depolarizing concentrations of potassium are represented as *filled symbols*; those obtained by boiling are represented as *open symbols. Circles* and *triangles* indicate data obtained from trout and goldfish inner ears, respectively. The number of extracted hair cells applied to the bioassay in each instance was estimated as follows: The number of hair cells in the otolithic organs of the goldfish was based upon published values (62,000 hair cells/5 cm goldfish; Platt, 1977). For trout, the number of hair cells in the otolithic organs was taken to be 5 times the value for the goldfish, on the basis of the data available for the saccule [35,000/trout saccule (A. Popper, personal communication) versus 7100/goldfish saccule (Platt, 1977)]. The estimated concentration of hair cells in the crude extracts was corrected for changes in sample volume in processing, dilution of the sample due to the chromatography step (based on measured dilution of sodium and glucose), and subsequent concentration of the chromatographic fractions before bioassay. Tissue concentrations were estimated from the mass of soft tissue of inner ears per fish, determined in pilot studies (4.9 mg for 7 cm goldfish, 10 mg for trout), multiplied by the number of fish processed. This value was then corrected for losses in processing, dilution during chromatography, and subsequent concentration before bioassay, as above. Separate scales of tissue concentration are used for trout and goldfish. The 2 *filled triangles with the highest values* represent dilutions of the peak fraction from one column; *all other points* represent bioassay values from different columns.

peaks of extracts prepared in these 2 ways (Fig. 5). In one instance where we obtained ears (for potassium treatment) from trout 1–3 hr after death, instead of a few minutes after death as in the rest of our studies, the apparent biological activity in the low-molecular-weight peak per hair-cell equivalent applied to the bioassay was lower by a factor of 3.

The sensitivity of the bioassay to changes in calcium (Fig. 1), and the ability of many substances to bind calcium and thus lower the free calcium concentration, prompted us to determine whether the biological activity of this low-molecular-weight peak was due to a reduction in free calcium in the fractionated extracts applied to the bioassay. In one column, fractionated extracts were adjusted for bioassay, tested for free calcium with a calcium electrode, and then bioassayed. The fractions in the low-molecular-weight peak of this column led to a 32% increase in the firing rate of the bioassay. A lowering of calcium to 1.0 mM was required to produce this activity (Fig. 1). Yet the free calcium of the bioassayed fractions in this low-molecular-weight peak was within 0.05 mM of the free calcium in artificial perilymph. Thus it is unlikely that the effect of the low-molecular-weight peak of activity from inner ear extracts is due to binding of calcium.

This peak of activity was not seen in tissue extracted with formic acid or ammonia, although this may have been due to technical problems associated with the use of these 2 extraction media. Acid treatment led to the dissolution of otoconia, with large amounts of salts that interfered with low-molecular-weight fractions. Ammonium as low as 200 μM inhibited the bioassay. Since boiling water did not introduce exogenous salts, it has been used in most of our recent studies of the substance(s) responsible for the low-molecular-weight peak of biological activity.

The success of several of the steps involved in processing extracts suggested further characteristics of the active substance(s) in the low-molecular-weight peak. For example, many of the procedures required concentrating the extracts by lyophilization. Since we detected activity after repeated lyophilization, the substance appeared to be nonvolatile. Activity was not apparently lost with repeated freeze–thaw cycles. Boiling the tissue served as an effective means of extracting activity, so this substance was not markedly heat-labile at neutral pH.

Characteristics of the high-molecular-weight excitatory peak

The biological activity of the high-molecular-weight excitatory peak of extracts obtained with high-potassium treatment also increased with the estimated number of extracted hair cells applied to the bioassay (correlation coefficient, 0.96; 5 samples). The activity of this peak from extracts obtained with boiling water, however, was not seen consistently.

The activity of this peak did not appear to be a nonspecific effect of the protein in these fractions. High-molecular-weight excitatory activity (21% increase in firing rate) was seen in the one extract we obtained from goldfish tissue with formic acid; the high-molecular-weight peak from this extract had a protein concentration below 0.1 mg/ml, the limit of detection of our routine protein assay. Activity of the high-molecular-weight peak was not significantly correlated with the amount of protein in the bioassayed fractions (20 samples: correlation coefficient, 0.38; $p > 0.05$).

This high-molecular-weight peak was further chromatographed with Sephadex G25, a gel that separates substances with M_rs between 1000 and 5000. This activity was slightly retained by Sephadex G25, suggesting a M_r of approximately 4000.

High-molecular-weight inhibitory activity was present in some fractions

In some extracts obtained with depolarizing concentrations of potassium from trout and skate, Sephadex chromatography showed, in addition to the 2 excitatory activities described above, an inhibitory activity eluting with the protein. This activity reached its maximum several hundred seconds after the excitatory effects of high-molecular-weight fractions. The delayed suppression of firing rate was gradually reversed, with a half-time of recovery of about 45 min. This activity was not retained

on Sephadex G25, suggesting that it may have been due to a substance with M_r greater than 5000.

Several known substances did not account for these biological activities

We have examined whether several known substances are responsible for the biological activities we found in extracts from inner ear tissue.

Glutamate and aspartate. Glutamate and aspartate are present in millimolar concentrations in all tissue, including the inner ear (Godfrey et al., 1976). They are known to affect activity in fibers innervating hair cells (Bobbin et al., 1981; Annoni et al., 1984), and have been suggested by some to be the transmitters at the synapse from the hair cell to the afferent neuron (Bobbin and Thompson, 1978). The biological activities described above, however, were not due to these amino acids. First, the effects of glutamate and aspartate on this bioassay were qualitatively different from those we saw in the 2 excitatory peaks. Unlike the essentially monophasic excitatory response seen in the bioassay in response to fractions from inner ear extracts, an inhibition of firing rate was always elicited by application of these 2 amino acids, sometimes preceded by a brief excitation (Fig. 6). With our protocol for data analysis the net effects of glutamate or aspartate were thus inhibitory when these amino acids were at high enough concentrations to affect firing rates. Furthermore, under the conditions we used for Sephadex G10 chromatography, glutamate and aspartate ran *between,* and were well separated from, the 2 excitatory peaks of biological activity, as has already been shown in Figure 4.

Glutamine and asparagine. These amino acids ran close to the low-molecular-weight excitatory peak obtained from Sephadex G10 chromatography and were excitatory at millimolar concentrations. The measured concentrations of glutamine and asparagine in these fractionated extracts, however, were more than an order of magnitude lower than the concentrations of these amino acids required to account for the excitatory activity of the low-molecular-weight peak. For example, a low-molecular-weight peak fraction in one column produced a 22% increase in the bioassay firing rate. Glutamine or asparagine concentrations of over 10 mM would be required to produce so large an effect. Yet the measured concentrations of glutamine and asparagine in this fraction were 30 and 120 μM, respectively.

GABA. The activity detected in these extracts is unlikely to have been due to GABA, a neurotransmitter that has been shown to excite nerve VIII fibers in the cat (Felix and Ehrenberger, 1977, 1982). The effect of GABA on our bioassay was similar to that described by Bobbin et al. (1985); it was a combination of slight excitation and inhibition, which was more prominent at low than at high doses. When the effects of GABA were analyzed with the standard analysis protocol, we found a modest depression of rate at low doses (10% at 50–300 μM) and no effect at 1–10 mM. Upon Sephadex G10 chromatography of brain extracts, GABA eluted between glutamate and glucose, before the low-molecular-weight excitatory peak.

Other known substances. To determine whether other substances possibly present in tissue extracts might account for the low-molecular-weight excitatory peak, we screened several with M_rs of less than a few hundred for their effects on the bioassay. Among the substances that were *not* excitatory at concentrations up to 5 mM were glycine, phenylalanine, alanine, tryptophan, leucine, isoleucine, histidine, methionine, arginine, cysteine, lysine, L-DOPA, met-enkephalin, *N*-acetyl aspartate, γ-glutamyl glutamate, γ-glutamyl glutamine, γ-glutamyl tyrosine, and γ-glutamyl phenylalanine. Thus these substances were unlikely to be responsible for either peak of excitatory activity upon Sephadex G10 chromatography.

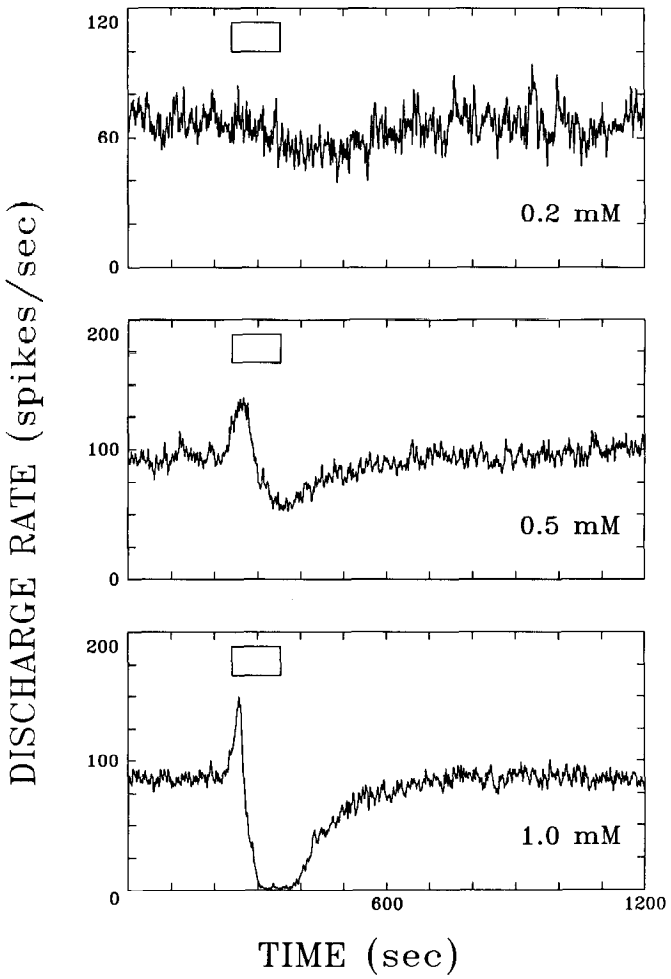

Figure 6. Effects of L-glutamate on the discharge rate of the bioassay. The discharge rate of fibers innervating hair cells of the lateral line organ in *Xenopus laevis* is plotted as a function of time for 3 different bioassay preparations. Solutions of L-glutamate were prepared in the perfusion solution and applied to the bioassay at the indicated concentrations during the time represented by the *open boxes.* These results are typical of those seen, although the relative proportions of excitation and inhibition varied among bioassays.

Discussion

In these studies we have looked in inner ear extracts for substances that affect the firing rates of afferent fibers innervating hair cells. This is a general approach to identifying substances that may have roles in neural function in the inner ear; it should be successful even if these substances are presently unknown or are not suspected to be neurotransmitters.

This type of approach should also be fruitful in studies of other neural systems where a source of starting tissue and a reliable bioassay can be found. For example, it is being applied by Obara and colleagues (Umekita et al., 1980) to studies of the neurotransmitter released by electroreceptor sensory cells. Although glutamate had been suggested as the afferent neurotransmitter at the electroreceptor afferent synapse (Steinbach and Bennett, 1971), Umekita et al. (1980) demonstrated the release of a neuroactive substance other than glutamate from electro-

receptor organs. Since it is thought that electroreceptors and hair cells might be related structures, it is possible that the substance detected by these investigators is similar to one of the excitatory substances we have detected in inner ear extracts.

Tissue sources

Fish inner ears provide a readily accessible source of large numbers of hair cells. The high proportion of hair cells in the tissue and the ease of dissection make this tissue a useful starting material for isolation studies. From the perspective of identifying neuroactive substances, the use of fish inner ears as starting material is a substantial advance over the starting material obtained in earlier experiments (Sewell et al., 1978) in which active substances were obtained in perfusates of guinea pig perilymph with volumes less than 60 μl. Although that earlier approach had the advantage that substance(s) released into the perfusates had a direct relation to acoustic function, the quantities of fluid collected were so small, the collection procedures so laborious, and the activity of the collected substance so low that purifying the active substances would not have been practical. By starting with fish inner ear tissue, we obtain enough starting material to make eventual identification of the active substances possible.

We tried treating tissue with depolarizing concentrations of potassium for 2 reasons. First, the relatively small number of presynaptic vesicles in hair cells (Liberman, 1980) suggests that the hair-cell neurotransmitter might be synthesized on demand, rather than stored in large quantities. Thus, depolarizing the tissue might generate larger quantities of active substances than would simple extraction. Second, only substances released into the bath would be collected with potassium treatment, reducing the amount of potentially interfering substances in the extract. We do not know if this potassium treatment succeeded in stimulating neurotransmitter release. The amount of low-molecular-weight excitatory activity in potassium-treated extracts and boiling water extracts was similar. It is possible that treatment with high potassium (which included centrifugation to separate fluid from tissue) merely served as a simple, room-temperature extraction step. The high-molecular-weight excitatory and inhibitory activities, however, were both greater in the potassium-treated extracts than in boiling water extracts. Some instability of these activities upon heating is a likely explanation of this latter finding.

It is possible that our initial tissue-processing steps led to the loss of active substances that were present in the tissue. Initial pilot studies did suggest that our cation-exchange treatment of crude tissue extracts did not greatly alter their activity in the bioassay. Quantitative comparison of crude versus treated extracts was difficult, however, because of the sensitivity of the bioassay to the cations present in the crude extracts. Further investigation of this tissue may best be done when the substances responsible for the activities in our treated extracts are identified.

Although we have extracted active substances from goldfish, skate, and trout, we have settled on goldfish for continuing work because they are routinely available from commercial suppliers in large numbers, can be delivered directly to the laboratory, and are easy to keep and store in the laboratory. The inner ears of trout were easily accessible, but trout are difficult to obtain and transport in large numbers. Skate extracts were very viscous and difficult to work with in the gel-permeation chromatography step.

Characteristics of the lateral line bioassay

Although fish ears provide a useful source of starting material, we have not found a bioassay based on fish acousticolateralis tissue that would be suitable for screening the multiple fractions generated during an isolation scheme. We thus turned to the *Xenopus* lateral line for a bioassay preparation. With this preparation it is possible to test 20 or more samples on the same afferent nerve fibers.

We have made 2 important adaptations to the *Xenopus* lateral line preparation reported by others (Bobbin et al., 1981) so that the preparation is more suited to the present investigations. First, the continuous perfusion of the inner surface of the skin minimizes mechanical artifacts in applying samples. Second, using immediately postmetamorphic frogs with thin skin allows rapid delivery of samples to the synaptic region of the neuromasts, as shown in the control experiments with cobalt.

Using the *Xenopus* preparation for a bioassay for samples derived from fish ears raises a possible problem in the species-specificity of the activities we have seen. We are working on the assumption that neurotransmitters active in the frog lateral line organ will be the same as or biologically similar to those in fish inner ears. Given the general conservation among vertebrates of the neurotransmitters identified thus far, this seems to be a reasonable working hypothesis. Once the active substances have been identified and purified, it will be possible to test this hypothesis directly.

High-molecular-weight active substances

Activity attributable to a high-molecular-weight excitatory substance was detected both in the inner ear and in the brain. This distribution is consistent with substances in efferent nerve fibers, with cell bodies in the brain and terminals in the inner ear. However, it is possible that the presence of the activity in the brain is unrelated to its presence in the inner ear. The variable activity seen in the high-molecular-weight peak from boiling water extracts may be due to an instability of the active substance upon heating or to adsorption of the excitatory substance onto denatured proteins.

In another study, we found that calcitonin-gene-related peptide (CGRP), a 37 amino acid peptide, can activate this *Xenopus* bioassay in nanomolar to micromolar concentrations (J. Adams, E. A. Mroz, and W. F. Sewell, unpublished observations). CGRP has been localized immunocytochemically in mammalian brain (Rosenfeld et al., 1983), in efferent fibers of the mammalian cochlea (Kitajiri et al., 1985; Schweitzer et al., 1985; Adams, 1986; Vetter et al., 1986), and in fibers innervating the lateral line of *Xenopus* (J. Adams, E. A. Mroz, and W. F. Sewell, unpublished observations). Preliminary studies indicate that CGRP-like immunoreactivity is present in goldfish inner ears (J. Adams, personal communication). The molecular weight of CGRP (approximately 4000, depending on the species) is similar to our estimate for the substance responsible for the high-molecular-weight excitatory peak. Thus CGRP or a CGRP-like peptide might account for the high-molecular-weight excitatory activity we have seen in extracts of fish ears and brain. Combined chemical and bioassay studies will be required to determine whether that is the case.

The high-molecular-weight inhibitory substance was detected only in extracts from trout and skate inner ears that had been treated with depolarizing concentrations of potassium. Its bi-

ological activity is striking, although the long duration of its effects would make it difficult to purify and identify.

Low-molecular-weight excitatory substance

The low-molecular-weight excitatory substance is a candidate as the hair-cell transmitter. We have extracted it from the inner ear; its effects on the bioassay are related to the number of hair cells extracted. We have not detected the low-molecular-weight excitatory substance in extracts of brain or muscle. This does not mean that the substance is not present in these tissues, but it does suggest that it is not as concentrated in these tissues as in hair cells. These experiments also indicate that the activity is not due to some substance that is a widely distributed metabolite.

This low-molecular-weight substance is stable upon boiling for at least 20 min, and to repeated freeze–thaw cycles and lyophilization. It is probably not a cation at neutral pH, since it is detected after treatment with cation-exchange resin. The activity in this peak cannot be accounted for by most common amino acids, including glutamate and aspartate, and it is not due to some substance interacting with calcium to lower the free calcium concentration in the applied sample. It is unlikely to be an artifact of the procedures used in extraction and separation, since it is not detected in brain and muscle extracts or in control procedures in which a salt solution was subjected to these procedures.

Implications for the neurochemistry of the inner ear

Glutamate and aspartate have been suggested as candidates for the afferent neurotransmitter, on the basis of their effects on discharge rate in afferent fibers (Bobbin and Thompson, 1978). The precise role of these amino acids in inner ear function is still unclear (Guth et al., 1981). Glutamate and aspartate are fundamental metabolic constituents of cells, and we found them in all tissues we examined, often in concentrations high enough to affect the bioassay. Our results do not rule out the possibility that glutamate or aspartate are hair-cell neurotransmitters. Our results do indicate, however, that inner ear tissue contains neuroactive substances other than those 2 amino acids.

The as-yet-unidentified auditory nerve-activating substance detected by Sewell et al. (1978) is another candidate for the hair-cell afferent transmitter; it also could not be accounted for by glutamate or aspartate. We do not know whether there is any relation between the auditory nerve-activating substance and the active substances we have described here. Our use of fish inner ears as starting material should eventually provide enough material to allow the determination of the chemical structures of the neuroactive substances. It should then be possible to determine whether these active substances are similar to the auditory nerve-activating substance collected by Sewell et al. (1978) or to any known neuroactive substances.

The active substances we have found in inner ear extracts are concentrated in hair-cell tissue and affect the firing rates of nerve fibers innervating hair cells. These substances thus meet 2 of the criteria for consideration as neurotransmitters. We cannot yet determine whether any of these activities are in fact due to neurotransmitters; if so, we cannot yet determine whether they are afferent or efferent neurotransmitters. Rather than speculate further on these issues, we believe it would be best to identify these substances, obtain them in pure form, and then determine the precise roles they play in inner ear function. Even if these active substances turn out not to be neurotransmitters, their presence in hair-cell tissue and their effects on afferent fibers suggest that they may be important in inner ear function.

References

Adams, J. (1986) Cells of origin of cochlear efferents in human. Abstracts of the Ninth Midwinter Research Meeting of the Association for Research in Otolaryngology, p. 5.

Annoni, J.-M., S. L. Cochran, and W. Precht (1984) Pharmacology of the vestibular hair cell-afferent fiber synapse in the frog. J. Neurosci. *4:* 2106–2116.

Bergmeyer, H. U., ed. (1974) *Methods of Enzymatic Analysis,* Vol. 4, 2nd ed. (D. H. Williamson, trans.), Academic, New York.

Bobbin, R. P., and M. H. Thompson (1978) Effects of putative transmitters on afferent cochlear transmission. Ann. Otol. Rhinol. Laryngol. *87:* 185–190.

Bobbin, R. P., S. C. Bledsoe, Jr., D. M. Chihal, and D. N. Morgan (1981) Comparative actions of glutamate and related substances on the *Xenopus laevis* lateral line. Comp. Biochem. Physiol. *69C:* 145–147.

Bobbin, R. P., S. C. Bledsoe, Jr., S. Winbery, G. Ceasar, and G. L. Jenison (1985) Comparative actions of GABA and acetylcholine on the *Xenopus laevis* lateral line. Comp. Biochem. Physiol. *80C:* 313–318.

Bradford, M. M. (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72:* 248–254.

Cooper, T. G. (1977) *The Tools of Biochemistry,* pp. 55–56, Wiley, New York.

Felix, D., and K. Ehrenberger (1977) The action of GABA and acetylcholine in the labyrinth of the cat. In *Inner Ear Biology,* vol. 68, M. Portmann and J. M. Aran, eds., pp. 147–154, INSERM, Paris.

Felix, D., and K. Ehrenberger (1982) The action of putative neurotransmitter substances in the cat labyrinth. Acta Otolaryngol. *93:* 311–314.

Flock, A., and D. M. K. Lam (1974) Neurotransmitter synthesis in inner ear and lateral line sense organs. Nature *249:* 142–144.

Flock, A., and I. Russell (1976) Inhibition by efferent nerve fibres: Action on hair cells and afferent synaptic transmission in the lateral line canal organ of the burbot *Lota lota.* J. Physiol. (Lond.) *257:* 45–62.

Gisselsson, L. (1950) Experimental investigations into the problem of humoral transmission in the cochlea. Acta Otolaryngol. (Suppl.) *82:* 3–78.

Godfrey, D. A., J. A. Carter, S. J. Berger, and F. M. Matschinsky (1976) Levels of putative transmitter amino acids in the guinea pig cochlea. J. Histochem. Cytochem. *24:* 470–472.

Good, N. E., and S. Izawa (1972) Hydrogen ion buffers. Methods Enzymol. *24:* 53–68.

Guth, P. S., M. Tachibana, and W. F. Sewell (1981) The pharmacology of the cochlear afferents and cochlear nucleus. In *Pharmacology of Hearing,* R. S. Brown and E. A. Daigneault, eds., pp. 99–136, Wiley, New York.

Harris, G. G., and A. Flock (1967) Spontaneous and evoked activity from the *Xenopus laevis* lateral line. In *Lateral Line Detectors,* P. Cahn, ed., pp. 135–161, Indiana U. P., Bloomington, IN.

Harris, G. G., and D. C. Milne (1966) Input-output characteristics of the lateral-line sense organs of *Xenopus laevis.* J. Acoust. Soc. Am. *40:* 32–42.

Kitajiri, M., T. Yamashita, Y. Tohyama, T. Kumazawa, Y. Takeda, Y. Kawasaki, T. Matsunaga, S. Grigis, C. Hillyard, I. MacIntyre, P. C. Emson, S. Shiosaka, and M. Tohyama (1985) Localization of calcitonin-gene-related peptide in the organ of Corti of the rat: An immunohistochemical study. Brain Res. *358:* 394–397.

Liberman, M. C. (1980) Morphological differences among radial afferent fibers in the cat cochlea: An electron microscopic study of serial sections. Hearing Res. *2:* 45–63.

Lowry, O. H., and J. V. Passonneau (1972) *A Flexible System of Enzymatic Analysis,* Academic, New York.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193:* 265–275.

Mroz, E. A., R. J. Roman, and C. Lechene (1982) Fluorescence assay for picomole quantities of ammonia. Kidney Int. *21:* 524–527.

Osborne, M. P., and R. A. Thornhill (1972) The effect of monoamine depleting drugs upon the synaptic bars in the inner ear of the bullfrog (*Rana catesbeiana*). Z. Zellforsch. Microskop. Anat. *127:* 347–355.

Platt, C. (1977) Hair cell distribution and orientation in goldfish otolith organs. J. Comp. Neurol. *172:* 283–297.

Rosenfeld, M. G., J. J. Mermod, S. G. Amara, L. W. Swanson, P. E. Sawchenko, J. Rivier, W. W. Vale, and R. M. Evans (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature *304:* 129–135.

Sand, O. (1975) Effects of different ionic environments on the mechano-sensitivity of lateral line organs in the mudpuppy. J. Comp. Physiol. *102:* 27–32.

Schweitzer, L. F., S. M. Lu, D. Dawbarn, and N. B. Cant (1985) Calcitonin gene-related peptide immunoreactivity in the superior olivary complex of the cat and rat: A specific label for the lateral olivocochlear system. Soc. Neurosci. Abstr. *11:* 1051.

Sewell, W. F., C. H. Norris, M. Tachibana, and P. S. Guth (1978) Detection of an auditory nerve-activating substance(s). Science *202:* 910–912.

Steinbach, A. B., and M. V. L. Bennett (1971) Effects of divalent ions and drugs on synaptic transmission in phasic electroreceptors in a mormyrid fish. J. Gen. Physiol. *58:* 580–598.

Umekita, S.-I., Y. Matsumoto, T. Abe, and S. Obara (1980) The afferent neurotransmitter in the ampullary electroreceptors: Stimulus-dependent release experiments refute the transmitter role of L-glutamate. Neurosci. Lett. (Suppl. 4): S7.

Vetter, D. E., J. C. Adams, and E. Mugnaini (1986) A dual efferent GABAergic projection to the rat cochlea. Soc. Neurosci. Abstr. *12:* 779.

Vinnikov, Ya. A., and L. K. Titova (1964) *The Organ of Corti,* p. 108, Consultants Bureau, New York.

Weakly, J. N. (1973) The action of cobalt ions on neuromuscular transmission in the frog. J. Physiol. (Lond.) *234:* 597–612.

Zimmerman, D. McG. (1979) Onset of neural function in the lateral line. Nature *282:* 82–84.