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# Effects of Furosemide on Distortion Product Otoacoustic Emissions and on Neuronal Responses in the Anteroventral Cochlear Nucleus

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## SUMMARY AND CONCLUSIONS

*1.* The objective of this study was to precisely evaluate the relationship between the threshold of neurons in the anteroventral cochlear nucleus (AVCN) and the properties of distortion product otoacoustic emissions (DPOAEs). Response areas of multiunit clusters in the AVCN and DPOAEs in the ear canal were measured alternately in the adult gerbil during furosemide-induced changes of the endocochlear potential. Stimulus frequencies of the probe tones for DPOAE measurement were in the range of $f_1$ = 1.7–7.6 kHz and $f_2$ = 2.0–9.0 kHz; the ratio $f_2$:$f_1$ was always 1.18. Stimulus amplitudes were varied in 5-dB steps from 30 to 80 dB SPL, with either equal amplitudes ($L_1 = L_2$) or unequal, with $L_1$ set 10 dB above $L_2$. Multiunit response areas were determined from cluster responses to a series of 100-ms tone bursts presented with a pseudorandom sequence in frequency and intensity.

*2.* Changes in the multiunit discharge properties after 50–75 mg/kg furosemide injection were as follows: the best frequency (BF) threshold increased from initial values in the range of 20–30 dB SPL to 50–80 dB SPL at 10–20 min postinjection and then recovered fully by 60–90 min. The spontaneous discharge activity decreased to zero before any changes in the frequency threshold curve were observed and did not return to initial values for several hours. Likewise, total discharge rates of stimulus elicited responses were reduced and tended to stay reduced even after BF threshold had fully recovered.

*3.* From the DPOAE measurements, the changes observed in the cubic distortion tone (CDT, $2f_1$-$f_2$) emission after furosemide injection were as follows: at high levels of the probe tones, changes in the emission intensities generally stayed within a 10-dB range. The CDT amplitudes for low stimulus levels, however, were typically reduced by up to 40 dB, but recovered (depending on the furosemide dosage) by ~60–90 min.

*4.* At low to moderate stimulus levels of 40–60 dB SPL, there was a near perfect, minute-by-minute covariation of the ear canal CDT amplitude and the BF threshold measured in the AVCN. A 10-dB increase in threshold was associated with a 5- to 7-dB decrease in the CDT emission.

*5.* The optimum stimulus parameter set for the noninvasive estimation of cochlear performance from the CDT response was for stimulus amplitudes $L_1$ = 50, $L_2$ = 40 dB SPL.

*6.* This experiment demonstrates that CDT emissions at low stimulus levels are very good predictors of the thresholds of cochlear afferents, but this validity is lost for BF thresholds greater than ~60–70 dB SPL.

*7.* The ear canal CDT amplitude is better correlated with the BF threshold sensitivity of neuronal response areas in the AVCN than with the spontaneous discharge rate or absolute above-threshold discharge rates.

## INTRODUCTION

Measurements of otoacoustic emissions in the ear canal have been increasingly used as a clinical and research tool to evaluate those characteristics of the mammalian cochlea that contribute to its extreme sensitivity and frequency resolution (for review, see Probst et al. 1991). However, to date, the relationship between these emissions and afferent response is largely circumstantial: agents that act to reduce or eliminate hearing sensitivity have been shown to also reduce or eliminate otoacoustic emissions (for review, see Patuzzi and Robertson 1988). It has not been shown that these two effects are intrinsically tied together—that both depend essentially on common biological processes—nor has the quantitative relationship between the strength of the emissions and the sensitivity of the auditory nerve fibers in the normal cochlea been established. For distortion product otoacoustic emissions (DPOAEs), the precise nature of this relationship must depend strongly on stimulus amplitude. It has been shown, for example, that the emissions for high level stimuli persist even after lethal anoxia (Rubel and Norton 1991; Schmiedt and Adams 1981; Whitehead et al. 1992a. 1992b).

The objectives of the present study are to establish the precise relationship between DPOAEs measured in the ear canal and the thresholds of neuronal responses measured in the anteroventral cochlear nucleus (AVCN). The Mongolian gerbil (*Meriones unguiculatus*) was chosen as an experimental animal because it has robust emissions (Brown 1987; Schmiedt and Adams 1981) and well-developed audition between 1 and 8 kHz (Ryan 1976), the frequency range where emissions are reported to be strongest. The experimental manipulation was to transiently interfere with cochlear functioning by applying the ototoxic diuretic, furosemide. It has been shown that furosemide severely reduces otoacoustic emissions to low level stimuli (Kemp and Brown 1984) as well as basilar membrane (BM) amplitude and tuning for low intensity stimuli (Ruggero and Rich 1991) and that it increases the excitation thresholds of auditory nerve fibers (Evans and Klinke 1982; Sewell 1984a–c). Results from a companion experiment that monitored the endocochlear potential (EP) and DPOAEs simultaneously in the same preparation indicated that although the vulnerable DPOAEs do decrease when the EP decreases sufficiently, they often recover completely while the EP is still subnormal (Mills et al. 1993a,b). It was therefore considered important in the present experiment to closely compare frequency threshold curves (FTCs) with vulnerable DPOAEs to establish whether the characteristics of neuronal firing properties were better correlated with the EP or with the vulnerable DPOAEs.

A major benefit of exploiting the ototoxicity of furosemide


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is that it only transiently impairs cochlear sensitivity. The plan of this experiment was therefore to record DPOAEs and the response properties from small groups of AVCN neurons over a period of 2–3 h. The effects of furosemide on neuron responsiveness could be compared with the effects on DPOAEs on a minute-by-minute basis during the period when cochlear sensitivity progressively decreased and during its recovery. Additionally, the reestablishment of the preinjection characteristics of both DPOAEs and neuronal thresholds indicates that the experimental manipulations had only affected the physiology of cochlear stimulus transduction and did not cause irreversible damage to some cochlear components.

To obtain an animal preparation with sufficient stability to monitor neuronal responses for several hours, response areas of multiunit ensembles were measured from the AVCN. Measurements of response characteristics in ''primary-like'' areas of the AVCN are known to be a very good indicators of cochlear function (Pfeiffer 1966a,b; Rhode and Smith 1986; Rose et al. 1974).

The ototoxic drug furosemide was applied by intraperitoneal injections. Intraperitoneal injections were preferred over intravenous injections because they caused more gradual changes in the EP, which allowed more data to be acquired on both threshold of neuronal responses and otoacoustic emissions during the transition time. For the same reason, once there was complete recovery of neuronal threshold from the initial injection, second and third injections were occasionally given.

## METHODS

Adult pigmented (agouti) Mongolian gerbils (*M. unguiculatus*), aged 3–6 mo, were used in the experiments (body weight 45–75 g). The animals were obtained from a commercial supplier (Tumblebrook Farms, Brookfield, MA) or from a colony kept at the experimental animal facility of the University of Washington Medical School. All experiments were performed in a double-walled sound-proof and echo-reduced chamber. The body temperature of the experimental animals was measured by a rectal probe and kept between 36.5 and 37.2°C by controlling the temperature of the recording chamber. All experimental procedures were approved by the University of Washington Animal Care Committee.

Because the results reported below were based on alternate recordings of neuronal responsiveness in the AVCN and on the measurements of DPOAEs in the outer ear canal, both experimental procedures are described in some detail. In general, each animal first underwent the experimental procedures necessary for stereotaxic electrophysiology in the AVCN. As soon as stable recordings were achieved, the sound delivery and recording systems for the measurement of DPOAEs were fitted to the ear canal and calibrated. Thereafter, both measurements could be performed in rapid alternation (each every 3–4 min) while the EP was temporarily lowered by injection of furosemide.

### *Animal preparation*

Before the experiment the animals were anesthetized with a combination of 15 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride (Xylazine). During the experiment, which lasted for 7–9 h, a constant level of anesthesia was maintained by hourly injections of one third of this initial mixture.

The pinna, surrounding skin, and outer portion of the external ear canal were removed unilaterally. The animal was then positioned in a stereotaxic device for electrophysiological recordings.

### *Electrophysiological recordings*

Using a motorized drill, two holes (300 $\mu$m diam) were drilled in the skull 2,500–3,000 $\mu$m caudal to the lambda suture, which corresponds to a position above the rostral third of the cerebellum. One drill hole, located 1,500 $\mu$m lateral to the midline, was used for positioning the indifferent electrode in the superficial cerebellum. Through the second drill hole, located in the midline, the recording electrode was inserted at an angle of 27–30° to the midsagittal plane. Glass pipettes filled with 3 M KCl (impedance 1–4 M$\Omega$) served as recording electrodes. In each experimental animal, the stereotaxic coordinates of the borders of the AVCN and of specific frequency representations within this nucleus were verified by online analysis of acoustic responsiveness acquired from multiunit ensembles. The organization of the ventral cochlear nucleus of the gerbil is comparable with that of other mammals (Cant 1992).

During the initial phase of the experiment, tone pulses were presented in ''near field.'' Pure tone stimuli used in the recording experiments (100-ms duration, 5-ms rise-fall time, 200-ms interstimulus intervals) were generated in an IBM-compatible laboratory computer (Compaq 386/20) equipped with a 12-bit D/A converter (Neuroboard; custom made). Stimulus presentation was performed in pseudorandom sequences of different frequencies and intensities. Each frequency-intensity combination was presented three times in a predefined frequency-intensity array (Fig. 1*A*). The sound transducer (Beyer DT-48) was mounted in a coupler with sound delivered through a 4-mm-diam tube ending close to the opening of the outer ear canal, ~5 mm from the animals eardrum. The output of the transducer was calibrated by placing a quarter-inch microphone (Bruel and Kjaer 4135) attached to a sound level meter (Bruel and Kjaer 2209) at the other end of the tube at a distance equivalent to the ear drum. (No correction was made for possible effects of the geometry of the outer ear canal on the sound field.) Computer-controlled attenuators were used to set the desired sound intensity. For this system, maximum intensity was 120 dB SPL from 0.2 to 15 kHz and 105 dB SPL up to 27 kHz.

Neuron responsiveness was measured in distinct isofrequency sheets of the AVCN, which were delineated electrophysiologically. Once a recording site with the desired best frequency (BF) and threshold was found, a higher resistance electrode (3–4 M$\Omega$) was used for monitoring the response. The exact position of this electrode was also verified by means of the recorded neuronal responsiveness. This electrode, which isolated three to five units in a ''cluster,'' was left in the same location for the remainder of the experiment. An Etymotic microphone/probe tube assembly (ER-1OB) was coupled to the ear canal with a short (0.5–1 cm) rubber tube. The acoustic signals from two transducers (Beyer DT-48) were fed into this closed cavity through small diameter tubes. For the measurements of the response properties of the multiunit clusters, tone bursts were delivered into the closed cavity by one of the transducers. The calibration of this closed system was subsequently confirmed by replacing one of the transducers with a calibrated probe microphone (Mills and Rubel 1994). Over the frequency range for which the ER-10B was used in these experiments (1–10 kHz), the error was found to be <2 dB. The calibration of the ''near field'' system used for the initial mapping could of course be compared with the closed system used for the remainder of each experiment by comparing the neuronal responses acquired with both, at the same site of the AVCN. There was always <10 dB difference in the BF threshold as determined by these two very different systems.

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### *DPOAE measurements*

For the DPOAE measurements, two tones (frequencies $f_1$ and $f_2$; $f_1 < f_2$) were generated by a signal processing board (Ariel) and delivered to the two transducers. Signals from the microphone were fed back through a preamplifier and band pass filter to the computer signal processing board and processed by fast Fourier transform.

Baseline DPOAEs were measured after the microphone was coupled to the ear canal. DPOAE data were recorded for stimulus frequencies of $f_1$ and $f_2$ in the range of 1.7–9.0 kHz, presented at a fixed $f_2$:$f_1$ ratio of 1.18. This ratio was used to relate our data directly to measurements from a companion study (Mills et al. 1993b). There the low ratio enabled also the analysis of the fifth-order distortion. The associated stimulus amplitudes $L_1$ and $L_2$ were either equal or unequal with $L_1 = 3.2\ L_2$ (10-dB difference). Before each sequence, the transducers were calibrated automatically with a wide spectrum signal. A single DPOAE record was begun with an initial amplitude $L_1 = 80$ dB SPL, with both amplitudes decreased simultaneously in 5-dB steps. The duration of averaging was automatically adjusted depending on the expected signal-to-noise level (extrapolated from the previous measurement), starting from 2 s up to a maximum averaging time of 20 s. The corresponding noise floor was maintained at approximately −20 dB SPL by this procedure. Amplitude and phase information were recorded for each stimulus presentation for the emission at the frequency $2f_1$-$f_2$, which may be termed the cubic distortion tone (CDT) emission. Closed cavity tests showed that the total instrumental distortion at the CDT frequency was ≥70 dB below the primaries.

### *Procedures and data analysis*

Once DPOAE measurements were begun, if the preinjection amplitude of the CDT emission was not ≥10 dB SPL with 50 dB SPL stimuli, the preparation was discarded. It was required that both the FTC and DPOAE be stable for 15 min before a furosemide injection was made. A single injection of furosemide (Lyphomed) at a dosage of 50–150 mg/kg was then given. Thereafter, sequences of DPOAE measurements were rapidly alternated with recordings of the units' response areas. The total time for one recording cycle (both FTC and DPOAE) typically was 3.5 min. After full recovery of the neural response threshold and CDT emission amplitude, additional injections of furosemide were occasionally given.

## RESULTS

Twenty-five gerbils were used. In five animals, stereotaxic coordinates for the approach of specific isofrequency areas in the AVCN were explored by recordings of multiunits and single isolated units. In one animal, the stability of response areas of AVCN multiunit ensembles over long recording times was tested, and eight animals were used to explore the effect of different furosemide dosages (50–150 mg/kg) on the response areas of multiunit ensembles and of single units. Two animals served as controls to evaluate the effects of furosemide on DPOAEs. In one of these, without furosemide treatment, the stability of the emissions under our specific experimental conditions were verified over a period of 3 h. In the other, which underwent furosemide treatment, the effects on emissions in a wider frequency range was tested by repetitive DPOAE measurements with frequencies of the primaries cyclically varied in 1-kHz steps in the range of 3.4–8 kHz. The effect of furosemide upon DPOAEs was found to be essentially the same across this frequency range. Nine animals were treated with furosemide, and both CDT emission and responsiveness of unit clusters in the anteroventral cochlear nucleus were measured. These nine animals formed the experimental group for the data reported below (Table 1). In two animals we tried to combine measurements of DPOAE and isolated single unit recordings but failed to get recordings that lasted throughout the entire treatment and recovery period. These data are not included in this report.

### *Initial frequency tuning curves*

Typical electrophysiological recording data from AVCN neurons are shown in Fig. 1*A*. In this figure the height of each bar represents the spike rate (logarithmic scale, maximum 40 spikes) during a single stimulus presentation of the given frequency and intensity combination. Note that each stimulus combination was presented three times. The multiunit neuronal response area, shown in Fig. 1*B*, was defined by the range of frequency-intensity combinations that caused cluster discharges to be at or above the 90% confidence level above spontaneous activity. Spontaneous activity was defined on each run by the discharge rate with maximal attenuation of the stimulus (e.g., in Fig. 1 by averaging responses at 10 dB SPL). The outline of the response area established the cluster's FTC, which was used to determine the BF threshold (Fig. 1*B*). Each successive contour line in Fig. 1*B* represents an increase in firing rate equivalent to the upper boundary of the 90% confidence interval of the discharge rate shown by the contour line just below. That is, the successive contour lines represent increasing discharge rates in steps defined by successive 90% confidence intervals. Changes in the multiunit responsiveness induced by furosemide injections were quantified based on these definitions. In control animals, without furosemide injections, the recordings were stable for 2.5–3 h. Fluctuations in BF threshold were ≤5–8 dB, even with intermittent injections of anesthesia.

### *Changes in units' BF threshold values after furosemide injection*

The effects of intraperitoneal furosemide injection on response areas were measured for multiunit clusters with BF between 1.3 and 23 kHz. In all cases, the changes observed were similar. The first effect of furosemide on neuronal activity, seen as early as ~5 min after the injection and before any observed change of the response threshold, was a drop of the spontaneous rate and of the tone evoked maximum discharge rate. A typical example is shown in Fig. 2. As illustrated by this response, the spontaneous activity of the units often recovered partly, as did the maximum discharge rate, but both measures did not recover to preinjection levels within 2–3 h, even when the units' response area or threshold appeared fully recovered (Fig. 2*C*). That is, there was complete recovery of threshold sensitivity at an overall reduced level of neuronal excitation.

Complete recovery of sensitivity with overall lower neuronal excitation can also be seen from discharge-rate-versus-stimulus-intensity curves obtained at different times after furosemide injection (Fig. 3). In this example, the maximum discharge rate was reduced by half (at 20 min postinjection)

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TABLE 1. *Effect of furosemide on CDT emission amplitude and on neuronal responses in the AVCN*

| Specimen | $f_1$:$f_2$, kHz | $2f_1-f_2$ Acoustic-Distortion Product, dB SPL | Best Frequency, kHz | Threshold Shift, dB SPL | Spontaneous Rate, spikes/s | Furosemide, mg/kg |
|---|---|---|---|---|---|---|
| MU22 | 5.9:7.0 | 13 to < −20 | 7.5 | 28 to >90 | 52 to 0 | 1 × 100 |
| MU23 | 2.4:3.0 | 12 to < −20 | 4.8 | 32 to >90 | 0.5 to 0 | 1 × 100 |
| | 4.1:4.8 | 11 to < −20 | | | | |
| | 6.7:8.0 | 18 to < −20 | | | | |
| MU24 | 1.7:2.0 | 12 to < −20 | 2.1 | 20 to >90 | 30 to 0 | 1 × 100 |
| MU25 | 5.1:6.0 | 16 to < −20 | 5.6 | 24 to >90 | | 1 × 100 |
| MU29 | 5.2:6.0 | 18 to < −20 | 1.4 | 18 to >90 | 1.0 to 0 | 2 × 75 |
| | | | | | | 1 × 50 |
| MU30 | 5.1:6.0 | 21 to < −20 | 1.8 | 22 to >90 | 40 to 0 | 1 × 100 |
| MU31 | 5.1:6.0 | 12 to −15 | 2.1 | 23 to 60 | 12 to 0 | 1 × 75 |
| MU32 | 5.1:6.0 | 18 to −12 | 5.2 | 20 to 50 | 170 to 0 | 2 × 75 |
| MU38 | 5.9:7.0 | 21 to < −20 | 5.5 | 18 to 83 | 230 to 0 | 1 × 75 |
| | | | | | | 2 × 50 |

CDT, cubic distortion zone; AVCN, anteroventral cochlear nucleus.

when threshold elevation was maximum (~70 dB SPL). Although this unit cluster showed complete recovery of BF threshold and nearly complete recovery of discharge rates to low stimulus intensity levels by 90 min, the maximum level of excitation remained reduced by one third.

The dynamics and magnitude of threshold deterioration and recovery depended on furosemide dosage. Injections of 50 mg/kg typically caused BF threshold elevations of 20–30 dB, and application of 75 mg/kg caused elevations of 30–70 dB and more. Injection of 100–150 mg/kg caused threshold shifts of 80 dB and greater. These high dosages were not often used in our experiments, because recovery was usually incomplete. The typical time span for recovery of the FTC also varied with dosage, from ~40–50 min for 50 mg/kg to ~60–90 min for 75 mg/kg.

### *Effect of furosemide on distortion product otoacoustic emissions*

The effects of furosemide on the magnitude of DPOAEs were measured in parallel with the effects on neuronal response characteristics. With our equipment, DPOAEs could be measured with test frequencies in the range of 1.7–9 kHz. Our initial experiments and parametric studies (Mills

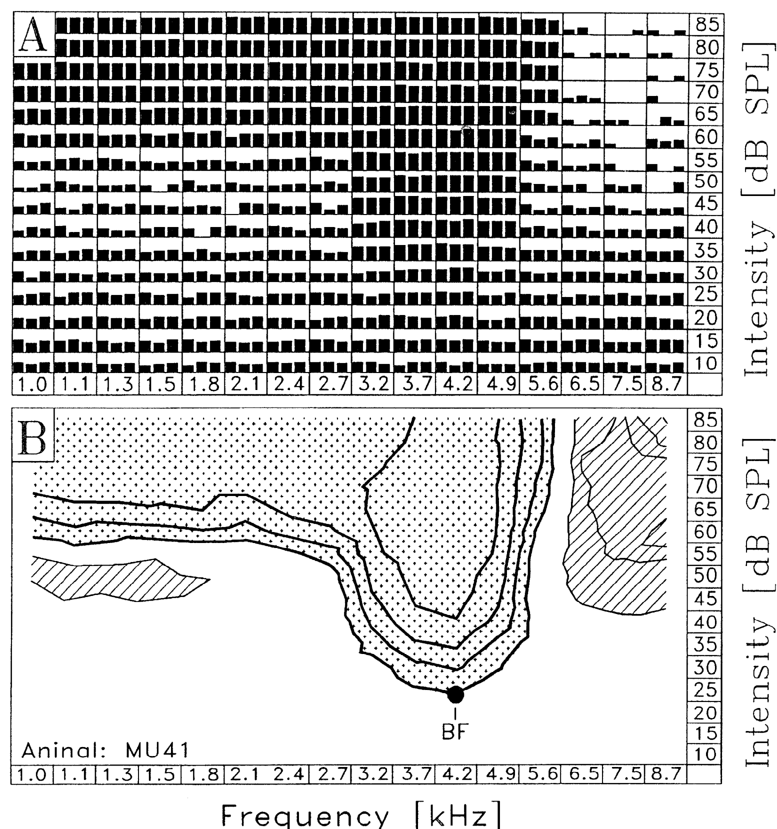


FIG. 1. Response area of a multiunit ensemble from the anteroventral cochlear nucleus (AVCN). *A*: display of the spike acquisition system used, showing a set of recordings in which 16 discrete pure tone stimuli (1.0–8.7 kHz) were presented at 16 different intensity levels between 10 and 85 dB SPL. Stimuli are presented 3 times at each frequency/intensity combination (100-ms duration, 200-ms interstimulus interval). Number of spikes evoked by each respective stimulus are indicated by the height of the bars (40 max). In the case presented, discharges recorded at the stimulus level of 10 dB SPL are used to determine the spontaneous activity level. *B*: isoactivation contours calculated for the above response area. Bottommost line encloses the stimulus conditions that, on a 90% confidence level, cause an increase of neuronal discharge rates above spontaneous firing rates, the minimum of this line defining BF threshold. The adjacent 3 lines enclose successive next-higher isoactivation contours that meet equivalent statistical criteria, e.g., for the calculation of the second isoactivation contour the level of responsiveness that defines the frequency threshold curve is taken as the base to identify stimulus conditions that, again on a 90% confidence level, lead to an increase of neuronal responsiveness.

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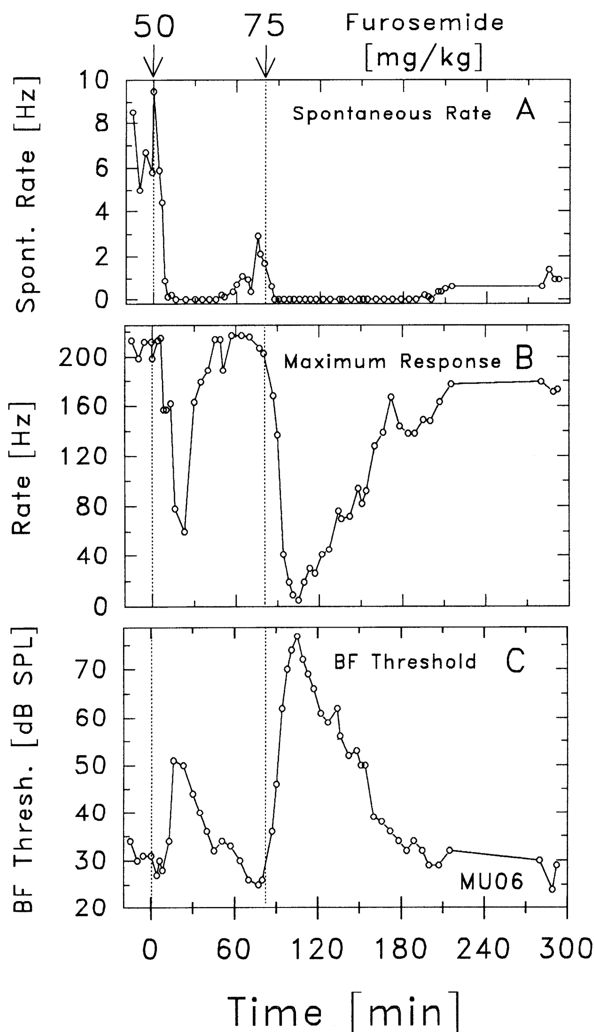


FIG. 2. Changes in neuronal response in AVCN for 1 animal after 2 furosemide injections (50 and 75 mg/kg) at times indicated by arrows at top (see Fig. 1 for definitions). *A*: spontaneous discharge rate vs. time; *B*: maximum tone-evoked excitation; *C*: BF threshold. Note that after furosemide injection, spontaneous discharge rate and maximum tone evoked discharge activity dropped rapidly and typically did not fully recover even after the original threshold values were reestablished. For example, the BF threshold had fully recovered at the time of the second injection (vertical dashed line) but the spontaneous rate had not.

and Rubel 1994) show that furosemide-induced changes in CDT emission amplitudes are virtually identical across this frequency range. Therefore frequencies of the test tones were generally chosen to yield the strongest preinjection CDT amplitude, which typically occurred for $f_2$ = 6–8 kHz ($f_2$:$f_1$ = 1.18).

After a furosemide injection of 50 mg/kg, the maximum change in CDT emission amplitude evoked by a high level stimulus was usually <10 dB. For example, in Fig. 4*A* the response for $L_1$ = 80 dB SPL varied between 40 and 50 dB over thè 52 min between preinjection measurement and recovery. In addition, changes in the CDT amplitude evoked by high level stimuli did not vary systematically with furosemide dosage, from 50 to 150 mg/kg.

A very different effect was seen with probe tones in the range of 30–60 dB SPL. As illustrated in Fig. 4*A*, both CDT emission ''threshold'' (defined by the noise floor of −20 dB SPL) and suprathreshold amplitudes were markedly affected by the furosemide injection. In fact, the CDT input-output function became nearly linear at the time of maximum effect (10 min after the injection).

In addition, at low levels of the probe tones, varying the dosage of furosemide caused variations in both the absolute amount of CDT reduction and the dynamics of recovery. With a 60-dB SPL stimulus level, the maximum reduction of the CDT emission after a 50-mg/kg injection of furosemide amounted to only 15–25 dB and was reached 8–15 min after injection. After an injection of 75 mg/kg, the maximum reduction was in the range of 30–40 dB and was typically reached somewhat sooner. The time span for recovery of the CDT to preinjection levels also depended on the furosemide dosage. Complete CDT recovery typically occurred by 30–50 min for 50 mg/kg furosemide (Fig. 5) and 60–90 min for 75 mg/kg furosemide. These values were typical for the first injection of furosemide. When a second injection was given upon complete recovery from the first injection, the onset of the effects and maximum CDT reduction generally occurred more rapidly and the recovery periods were somewhat prolonged.

There are two different ways to examine the reduction in the CDT emission amplitude. As illustrated in Fig. 4*A*, a change in the growth function from the preinjection level to the growth function at 15 min postinjection can be considered a decrease of 21 dB in the CDT emission amplitude at a stimulus level of $L_1$ = 40 dB SPL (the decrease ''d'' noted in Fig. 4*A*), or it can be considered equivalent to a ''threshold increase,'' that is, the amount of increase in stimulus amplitudes required to reestablish the preinjection CDT amplitude (the increase ''t'' noted in Fig. 4*A*). The example shown would require an increase of 8 dB in $L_1$, with the same increase required in $L_2$, because these growth functions are presented for a fixed stimulus amplitude ratio. The general relationship between these two parameters is represented in Fig. 4*B* for the same growth functions as shown in Fig. 4*A*. It can be seen that a greater change occurred in the CDT amplitude at a given stimulus level than in the associated

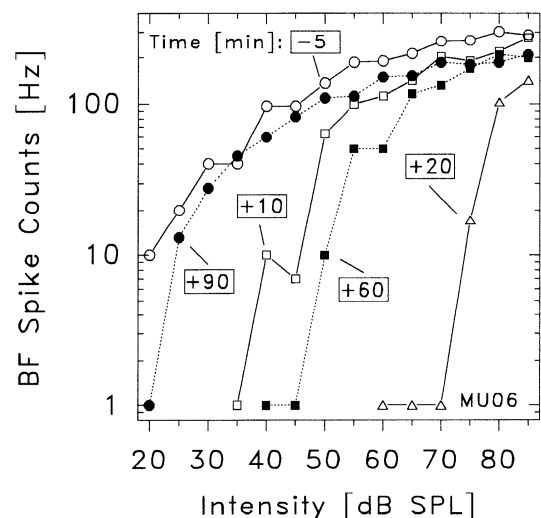


FIG. 3. Changes in the discharge rate vs. stimulus intensity function after injection of 75 mg/kg furosemide. ○, before injection (−5); □, 10 min; △, 20 min; ■, 60 min; ●, 90 min after furosemide injection.

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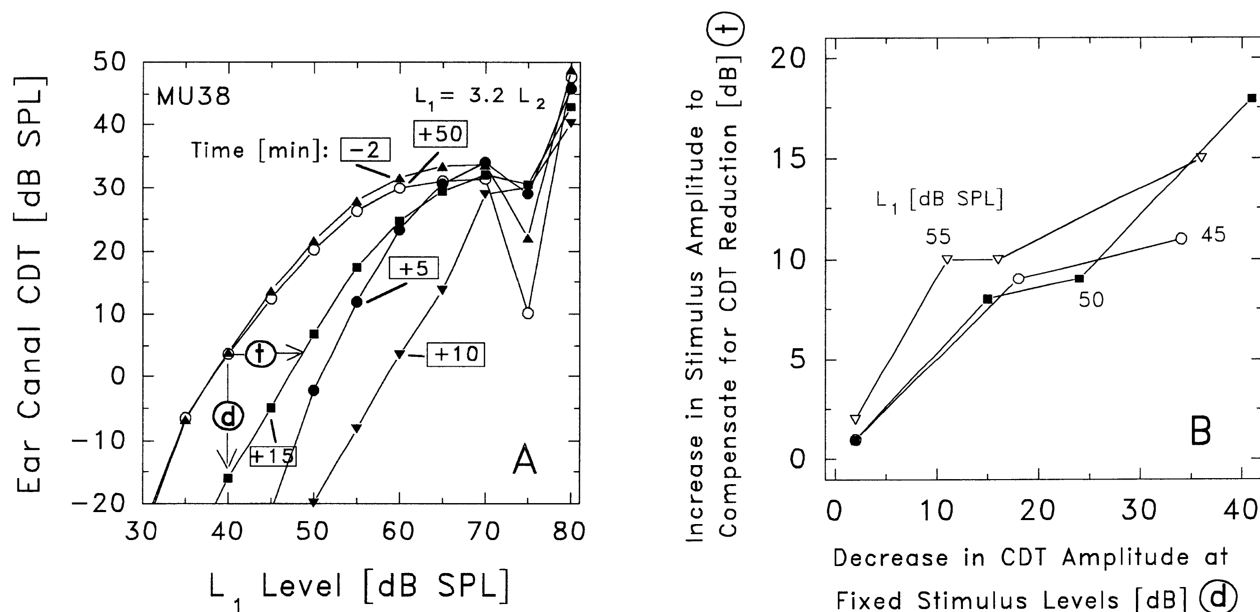


FIG. 4. Variation of cubic distortion tone (CDT) emission after injection of 50 mg/kg furosemide, as a function of the stimulus levels of the primaries. Frequencies of the test tones are $f_1$ = 6.8 kHz, $f_2$ = 8 kHz. Numbers in the graphs (e.g., −2) give the time in minutes relative to the furosemide injection. *A*: CDT emission amplitude vs. stimulus level (growth function) with the parameter the time since injection. Change at a given time (e.g., 15 min postinjection) can be considered either as the decrease in CDT amplitude at a given stimulus level (i.e., the decrease denoted by "d") or the amount of stimulus increase required to obtain the same CDT amplitude (the increase denoted by "t"). *B*: 2 quantities plotted against each other for several different values of the original stimulus amplitudes.

threshold-equivalent increase. This comparison also shows that at moderate stimulus levels the observed decrease in DPOAE amplitude is linearly related to the increase in stimulus amplitude required to compensate the CDT reduction, that is, the analysis of furosemide-induced CDT changes will give equivalent results irrespective of whether the actual change of CDT amplitude at a given stimulus level is presented (as usually done in the present experiments), or the effect on CDT emission amplitude is shown on the basis of an equivalent threshold criterion. Because both types of analysis are equivalent, it is also reasonable to directly relate either to changes in neuronal thresholds.

### *Comparison of changes in neuronal response properties with changes in DPOAEs*

Nearly simultaneous measurements (within 2–3 min) of changes in CDT emission amplitude and changes in the response properties of AVCN multiunit clusters were acquired in nine experiments (Table 1). In the experiment shown in Fig. 5, furosemide was injected three times. The BF of the units was 5.5 kHz, and the initial injection of 75 mg/kg caused a multiunit BF threshold increase from 26 to 82 dB SPL. Complete threshold recovery occurred within 70 min. Two subsequent injections of 50 mg/kg each caused transient BF threshold increases of 22 and 42 dB, respectively (Fig. 5*A*). Alternating measurements yielded changes of the CDT emission in response to moderate stimulus levels that occurred in synchrony with changes of FTC threshold (Fig. 5*B*). For probe tone intensities of $L_1 \times L_2 = 60 \times 50$ dB SPL, maximum CDT reductions amounted to 40, 15, and 29 dB, respectively, for the three injections. For probe tone intensities of $L_1 \times L_2 = 50 \times 40$ dB SPL, the maximum reductions of the CDT emission after the three furosemide injections were 34, 32, and 42 dB, respectively. Changes in the CDT amplitude could not be completely quantified for lower intensities of the probe tones, because the intensity of the emissions were reduced below the noise floor of the recording system (about −20 dB SPL) at maximum effect. In Fig. 5, it should also be noted that the CDT changes with higher probe intensities (80 × 70 dB SPL) were considerably smaller.

To directly compare the temporal dynamics of altered neuronal threshold values and variations in the CDT amplitudes, data from Fig. 5, *A* and *B*, were normalized to the maximum changes that occurred and superimposed (Fig. 6). This figure demonstrates that after furosemide injection, both the CDT emission levels and the BF thresholds covaried on a minute-to-minute basis. Data from this same animal are replotted in Fig. 7 to directly compare the values of CDT emission obtained for different levels of the probe tones with the corresponding BF threshold values. The fact that CDT amplitudes for high stimulus levels ($L_1 \times L_2$= 80 × 70 dB SPL) showed relatively little change after furosemide injections is evidenced by the shallow slope of the uppermost regression line (b = 0.13). For moderate and low intensities of the probe tones ($L_1 \times L_2$ between 60 × 50 and 40 × 30 dB SPL), however, a linear regression analysis shows that a FTC threshold increase of 10 dB was correlated to a decrease in CDT emission magnitude in the range of 5–7 dB (b = −0.5 to −0.7).

The fact that CDT emission versus BF threshold values were more scattered when CDT values showed maximum reduction is probably due to the inevitable 2–3 min difference between a DPOAE measurement and the acquisition of neuronal responses. Close to their maximum changes,

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both the CDT levels and neuronal thresholds were changing rapidly, and it was not possible to interpolate accurately to obtain exactly ''corresponding'' measurements.

The relation between changes in CDT emission amplitudes and changes in neuronal thresholds are shown in Fig. 8 for four additional experiments, all for stimulus levels $L_1 \times L_2$ = 50 × 50 dB SPL. In these animals, threshold shifts of 10 dB were correlated with CDT emission changes between 5.1 and 6.2 dB. In all cases, the correlation was over 0.85, even though the range was limited in some cases (e.g., MU32).

## DISCUSSION

### *Effects of furosemide on AVCN neuronal responses*

In these experiments, the effect of intraperitoneal injection of ~75 mg/kg furosemide was typically found to have the following effects on primary-like responses recorded from unit clusters in AVCN. First, the spontaneous activity and gross discharge rate decreased. The multiunit neuronal threshold response, defined as the threshold for evoking activity reliably above the spontaneous rate as a function of frequency, did not appear to change significantly during this initial effect. The BF threshold then rose sharply from 20 to 80 dB SPL. The maximum furosemide effect usually occurred ~20 min postinjection, and then the neuronal sensitivity began to recover. Essentially, complete threshold recovery was usually found by 60–90 min postinjection. However, the spontaneous rate and maximum discharge rate did not recover completely until several hours later, if ever.

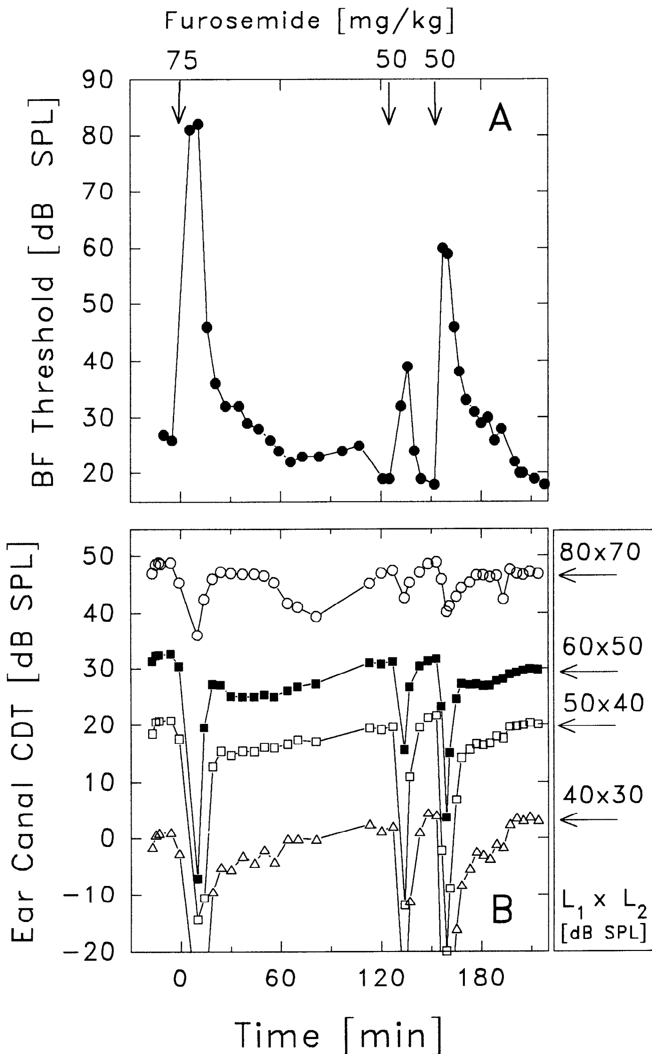


FIG. 5. Measurements of changes of BF threshold in the AVCN and ear canal CDT emission amplitude in one animal. *A*: change in neuronal threshold after 3 different injections of furosemide (75, 50, and 50 mg/kg) at times indicated by arrows on the top of the graph. *B*: amplitudes of CDT emission measured at different levels of the test tones. Respective intensity values ($L_1 \times L_2$) are indicated by arrows to the right of the graph.

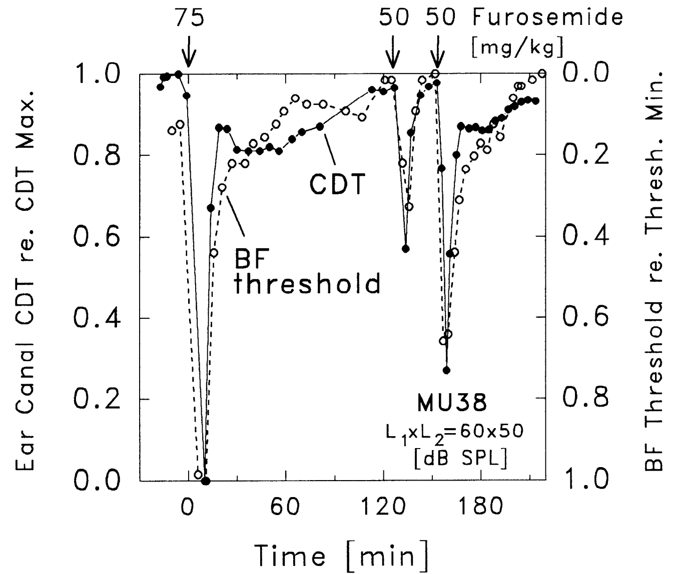


FIG. 6. Comparison of the dynamics of AVCN neuronal threshold shifts and the ear canal CDT amplitude after 3 different furosemide injections (same animal as Fig. 5). In this graph both sets of data are normalized to the maximum changes observed during the experiment (normalized logarithmic scales). Emissions are reported for stimulus levels $L_1 \times L_2$ = 60 × 50 dB SPL because at these stimulus intensities the CDT amplitudes never dropped below the noise floor of −20 dB SPL. CDT (solid line) refer to the left ordinate; BF threshold (dashed line) refer to the right ordinate.

The results of the present study generally agree quite well with those previously obtained by direct measurement of eighth-nerve responses after furosemide injections (Comis et al. 1981; Evans and Klinke 1982; Sewell 1984a–c). However, the present results on spontaneous discharge rates and maximum discharge rates differ somewhat from those reported by Evans and Klinke (1982), who found that decreases in these rates were more variable than found here. In their experiments, however, a number of smaller intravenous dosages of furosemide were used. They report that some injections had no apparent effect, whereas the same dosages a few minutes later had a large effect. The sequence of events in the cochlea may well have been affected differently by this procedure than by the large single intraperitoneal injection of furosemide used in the present study.

### *Covariation of specific neuronal thresholds and DPOAE characteristics*

The major result from this study is that the amplitude of the CDT emission to moderate stimulus level probes directly covaries with the sensitivity of neuronal unit clusters in the

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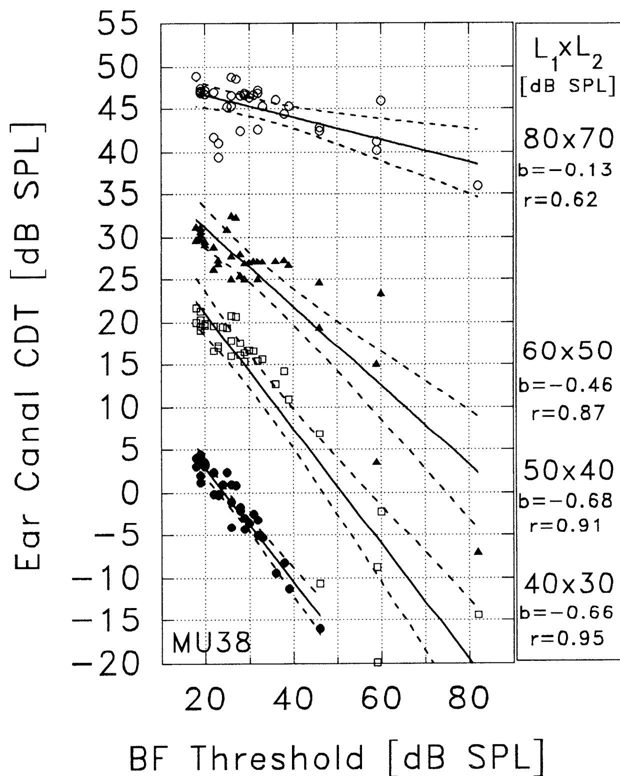


FIG. 7. Changes in the ear canal CDT amplitude compared with changes of neuronal BF threshold values in the AVCN, recorded from a single animal with 3 furosemide injections (see Fig. 5). CDT amplitudes at different primary levels are shown by different symbols and regression lines. For each set of data the regression lines are indicated on the right (assuming linear regression, $r$ = correlation coefficient, b = slope, dashed lines enclose 99% confidence intervals).

primary-like areas of the AVCN. Specifically, for DPOAE stimulus levels below 60 dB SPL, a 10-dB increase in BF threshold is reliably associated with a 6- to 7-dB decrease in the CDT emission amplitude. There is a very close, minute-by-minute covariation of the CDT amplitude and BF threshold, even over an experiment lasting several hours and including multiple injections.

A companion experiment (Mills et al. 1993a,b) showed that the vulnerable DPOAE, although initially reduced by the change in EP, recover well before the EP does. At 60–90 min postinjection (75 mg/kg), for example, the EP is typically only 50 mV, contrasted to initial levels of 90 mV in these animals. In contrast, the emissions and BF thresholds have typically recovered completely (e.g., Fig. 2). Therefore, the results obtained here infer that the thresholds of cochlear afferents covary more closely with CDT emissions than they do with the EP. The spontaneous rate and maximum discharge rate, on the other hand, do appear to covary over time more with the EP than with the DPOAE. Put another way, both cochlear sensitivity and the vulnerable CDT amplitudes appear to recover despite the persistence of a subnormal EP. They appear to be much more closely correlated with each other than either is to the EP. The obvious implication of this fact is that the mechanisms that give rise to these seemingly disparate measures of cochlear function must be intimately associated. That is, the mechanisms that underlie sensitivity must also underlie the production of DPOAEs in response to low-to-moderate level stimuli (Kemp 1978, 1986). The underlying mechanisms must be interfered with by an abrupt change in EP but must be able to recover normal function despite persistence of a subnormal EP.

Covariation of these central neuronal response properties and of CDT emissions does not come as a surprise. This result is expected from what we know about the relationship between BM motion and neuronal activity on the one hand and between BM motion and DPOAEs on the other hand. It has been shown repeatedly that the tuning of the BM is almost identical to that of hair cells and of cochlear afferents (Dallos 1985, 1992; Patuzzi and Robertson 1988; Robles et al. 1986; Ruggero 1992; Russell and Sellick 1978; Sellick et al. 1982, 1983). Frequency-specific properties of auditory nerve fiber responses, like two-tone rate suppression, have counterparts in BM responses (Arthur et al. 1971; Robles et al. 1986, 1991; Sachs and Kiang 1968). Furthermore, vulnerability of auditory nerve fiber frequency tuning, sensitivity, and nonlinearities relate to vulnerability of BM motion (Patuzzi et al. 1989; Robertson 1976; Robertson and Johnstone 1981; Robles et al. 1989; Ruggero and Rich 1991; Schmiedt and Zwislocki 1980). It is hypothesized that a DPOAE component at the ear canal consists of a summation of mechanical distortion products distributed along the BM/organ of Corti complex (Nuttall et al. 1990; Robles et al. 1991). Thus they would be expected to change in similar ways as BM and eighth nerve response nonlinearities. It was nonetheless necessary to directly determine the quantitative relation between neuronal response characteristic and DPOAE based on nearly simultaneous measurements of both. This analysis also revealed some unexpected differences between the changes in neuronal response properties threshold and DPOAEs. The interpretation of these differences is discussed next.

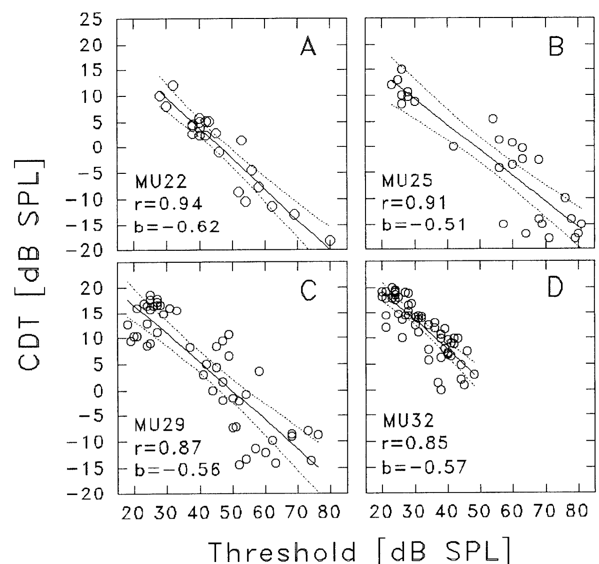


FIG. 8. Changes in the ear canal CDT amplitude related to changes of AVCN neuronal BF thresholds. $A$–$D$: data for 4 different animals; stimulus levels 50 × 50 dB SPL in all cases. $C$ and $D$: data from successive furosemide injections. Linear regression analysis indicates that CDT emission amplitudes are decreased by 5–6 dB with neuronal threshold increases of 10 dB.

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*Interpretation*

When the present findings are considered in the light of the results of the companion study (Mills et al. 1993a,b) and direct measurements of BM motion (Ruggero and Rich 1991), the following interpretation appears most likely. Cochlear function—in terms of BM motion—appears to be normally at a position of a ''relative maximum'' as a function of the EP. That is, when furosemide produces an initial decrease in the EP, there is little change in the vulnerable CDT amplitudes or, presumably, in the BM motion itself (Mills et al. 1993a,b). The reduced EP, however, implies a reduced voltage across the inner hair cell transduction channels. This change would be likely to produce a reduction in the spontaneous rate and maximum discharge rate of the cochlear afferents. There is, nonetheless, no observable change in the BF threshold at the level of AVCN units during this initial decrease of EP and of spontaneous discharge rates.

With an additional decrease of the EP, BM motion is changed. The major effect is the loss of the sharp peak in the motion near the BF for low amplitude stimuli (Ruggero and Rich 1991). This loss causes an increase in neuronal BF threshold. There is an additional increase in neuronal threshold because of the effect of the additional EP decrease on inner hair cell dynamics, which we discuss below. After maximum effect, cochlear functioning in terms of the BM motion appears to adapt to the change in EP and recovers fully with a subnormal EP (Mills et al. 1993b). This recovery is evidenced in the essentially complete recovery of the neuronal thresholds and the emissions. In contrast, the spontaneous rate and gross discharge rate do not recover completely until the EP recovers completely, which can take several hours with intraperitoneal injections (Mills et al. 1993b).

The initial increase of BF threshold for furosemide injections of 75–100 mg/kg is 50–70 dB or more (Table 1). Although speculative, it is of interest to estimate the contributions to this decrease that can be attributed to different mechanisms. If the furosemide dosage is sufficient to completely ''linearize'' the BM response by inactivation of outer hair cells (OHCs), this would cause an increase of threshold of ~35–40 dB in the chinchilla, starting from a threshold of ~20 dB SPL (see Figs. 1 and 2 of Ruggero and Rich 1991). In addition, there are direct effects of the change in EP on inner hair cell transduction. An intraperitoneal dosage of 75–100 mg/kg will cause an EP decrease at maximum effect of 60–90 mV (Mills et al. 1993b). This decreases the voltage driving the transduction current into the inner hair cell by a factor of two to three, accounting for an approximate 6- to 10-dB increase in threshold. However, it is likely that there are more important effects due to hyperpolarization of the inner hair cell intracellular resting potential caused by the decrease in EP. From in vivo asphyxia measurements, Nuttal (1984) estimated that the inner hair cell intracellular voltage dropped 1 mV for each 10-mV decrease in EP. Because the rate of release of synaptic vesicles appears to depend exponentially on the membrane voltage (Sewell 1984c), even a small decrease in the membrane resting potential will cause a substantial reduction of transmitter release. From Sewell's data, we estimate that an inner hair cell membrane voltage decrease of 6–9 mV would cause a change in transmitter release equivalent to a threshold increase of 18–26 dB. Adding the 6- to 10-dB contribution expected because of the decreased transduction current, the total increase in threshold due to effects of the EP decrease on inner hair cells alone is ~24–36 dB.

These effects are more than enough to account for the observed increase in AVCN neural threshold caused by a sharp drop in EP. In general, then, about half of the observed threshold increase can be attributed to a loss of the peak BM response due to the interruption of OHC/cochlear amplifier function and about half can be attributed to direct effects of the EP decrease on the inner hair cell transduction process itself.

*Distortion product otoacoustic emissions as a noninvasive tool for measurement of cochlear sensitivity*

There are several reasons why researchers, who are investigating the physiology of the cochlea, and clinicians, who are in need of a sensitive method for the diagnosis of hearing loss of cochlear origin, are specifically interested in distortion product otoacoustic emissions: *1)* DPOAEs, especially the CDT at $2f_1$-$f_2$, can be reliably measured in all individuals with a normal cochlea and a normal conductive apparatus, *2)* DPOAEs can be measured by noninvasive procedures, *3)* the success of the measurement does not depend on the cooperation of the subject and can be accomplished on anesthetized animals, and *4)* the results obtained can provide highly frequency specific information (for review, see Probst et al. 1991).

There is considerable experimental evidence that makes it plausible to assume that otoacoustic emissions in mammals are intrinsically related to active cochlear processes and specifically to outer hair cells acting as a ''cochlear amplifier'' impinging force on the BM during the process of mechanoelectrical stimulus transduction (Davis 1983; Kemp 1978, 1986; Neely and Kim 1983, 1986; Patuzzi and Robertson 1988). Indeed, stimulus-induced force generating motility has been shown in in vitro preparations of OHCs (Ashmore 1990; Brownell 1990; Brownell et al. 1985; Zenner et al. 1987). Furthermore, direct measurements of BM motion, using the Mössbauer technique or Doppler-shift laser velocimetry, reveal high amplitudes of displacement and narrowly tuned frequency response at moderate acoustic stimulus levels (40–60 dB SPL) (Johnstone et al. 1986; Rhode 1971; Robles et al. 1986; Ruggero and Rich 1991; Sellick et al. 1982). The performance of the putative cochlear amplifier is also reflected in the frequency response of receptor potentials of both outer and inner hair cells (Cody and Russell 1987, 1988) and in the discharge activity of auditory nerve fibers (Evans 1972; Liberman and Kiang 1978; Schmiedt 1989).

Impairment or damage to the cochlear amplifier has comparable effects on the BM responses, on the activity of auditory nerve fibers, and on otoacoustic emissions. For example, when the otoacoustic emissions are abolished as a consequence of outer hair cell destruction (Brown et al. 1989; Schmiedt 1986), the amplitude of BM vibration at moderate stimulus levels is also reduced (Johnstone et al. 1986) and the threshold of auditory nerve fiber responses is increased

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(Kiang et al. 1976; Liberman and Kiang 1978). Furthermore, the CDT is vulnerable to cochlear damage when assessed by psychoacoustical (Smoorenburg 1972) and neurophysiological methods (Siegel et al. 1982).

However, the quantitative relationship between CDT emission amplitude and the sensitivity of auditory afferent responsiveness has not been previously described, because most of the measurements mentioned above were based on the destruction of OHCs, which is an all-or-none intrusion of the cochlear system. In the present work our intent was to transiently interfere with the cochlear function without causing morphological damage to the organ of Corti. This was accomplished by using the ototoxic drug furosemide that, in a dose-dependent fashion, temporarily reduces the EP (Forge and Brown 1982; Kusakari et al. 1978; Rybak and Morizono 1982; Syka and Melichar 1985). Furosemide was used in earlier experiments to separately explore the effect of EP reduction on the DPOAEs (Kemp and Brown 1984) and on cochlear eight nerve responses (Evans and Klinke 1982; Sewell 1984a–c).

When comparing the effect of furosemide on the CDT emission amplitude with its effects on neuronal response thresholds, it is important to realize that the determination of a response threshold is based on the establishment of a response criterion that must be met or exceeded by the measurement, whereas normal useage of DPOAE involves measurements of response levels acquired at fixed stimulus levels. For primary levels between 45 and 55 dB SPL, there is typically a compensation level difference of 5 dB in tone stimuli for each 10-dB drop of the cubic distortion tone amplitude (Fig. 4*B*). Because this relation is fairly constant across the entire range of observed levels of CDT emission from +20 to −20 dB SPL, it is equivalent to compare these level measurements with the sensitivity of cochlear afferents.

It might be promising to establish similar relations for other mammalian species, including humans, as a noninvasive tool for the frequency-specific evaluation of cochlear sensitivity. However, the specific relation between the CDT emission amplitude and cochlear sensitivity will have to be established in each species separately. For example, in two rodent species for which we have both well-documented audiograms and DPOAE measurements, the absolute amplitude of the CDT emission is larger in the Mongolian gerbil than in the guinea pig (Brown 1987), although the gerbil's hearing sensitivity in the same frequency range is apparently ~15 dB worse than in the guinea pig (Prosen et al. 1978; Ryan 1976). Our results do show that, for gerbils, the CDT resulting from stimulus levels of about $L_1 = 50$ and $L_2 =$ 40 dB SPL gives a reliable prediction of the cochlear afferent threshold sensitivity. The specific properties of this relationship are not known for other species.

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