Evaluation of cochlear function in an acute endolymphatic hydrops model in the guinea pig by measuring low-level DPOAEs

Valk WL, Wit HP, Albers FWJ.
Evaluation of cochlear function in an acute endolymphatic hydrops model in the guinea pig by measuring low-level DPOAEs.
Hearing Research 2004;192:47-56.
Introduction

Since Menière’s disease was first documented (Menière, 1861), its cause remains an enigma. The chronic illness is characterised by disabling attacks of vertigo, fluctuating subacute hearing loss, tinnitus and a sensation of aural fullness. An endolymphatic hydrops, which is an excess of endolymph volume, has been generally accepted as the basic histopathological substrate (Hallpike and Cairns, 1938). Studies on experimentally induced hydrops constitute a useful model for Menière’s disease. Over the years, the most extensively studied model has been the surgical ablation of the endolymphatic sac in the guinea pig. This model, first described by Kimura and Schuknecht (1965), results in a slowly developing chronic endolymphatic hydrops.

An experimental model of an acute endolymphatic hydrops involves the micro-injection of artificial endolymph (Jin et al., 1990; Salt and DeMott, 1997; Kakigi and Takeda, 1998; Wit et al., 2000). This model eliminates the secondary effects of a surgically induced chronic hydrops and provides a useful research tool for investigating the immediate effects of an endolymphatic hydrops on the inner ear. To date, evaluation of cochlear function has only been performed by electrophysiological measurements under these experimental conditions (Jin et al., 1990; Sirjani et al., 2003). The present study was designed to register the effects on cochlear function by recording of low-level distortion product otoacoustic emissions (DPOAEs).

Twenty-five years ago Kemp (1978) demonstrated that the cochlea produced otoacoustic emissions (OAEs). These OAEs were measured in the external ear canal as responses that the cochlea generated in the form of acoustic energy. Nowadays, otoacoustic emissions are widely accepted to reflect the integrity of cochlear micromechanical processes in general, and outer hair cell (OHC) function in particular (Kim, 1986).

Through their association with OHCs, DPOAEs have been linked to the active processes responsible for the cochlea’s sensitivity and frequency selectivity. OHCs act as the cochlear amplifier by their unique motor property: the OHC motility (Brownell et al., 2001). As the functionality of outer hair cells is very sensitive to environmental factors, DPOAEs are now widely used to assess cochlear function in experimental models detecting even minimal OHC dysfunction. The $2f_1 - f_2$ DPOAE is the most prominent and most frequently used DPOAE. For example, DPOAEs were used for assessment of gentamicin (Shi and Martin, 1997) and kanamycin-furosemide-induced ototoxicity (Alam et al., 1998), cochlear damage caused by acoustic overstimulation (Emmerich et al., 2000) and genetic OHC dysfunction in the guinea pig (Horner et al., 1985). Several researchers state that measurement of DPOAEs is a useful tool for monitoring of cochlear function in different stages of Menière’s disease (Cianfrone et al., 2000; Kusuki et al., 1998; Magliulo et al., 2001; Perez et al., 1997). Specifically, with the aim to investigate the involvement of cochlear outer hair cells in cochlear disfunction in patients with an endolymphatic hydrops.

The chronic endolymphatic hydrops animal model has already been investigated extensively using DPOAEs (Martin et al., 1989; Horner, 1991; Magliulo et al., 1996; Okubo et al., 1997). In these studies reduced DPOAEs were found in hydropic cochleas and DPOAEs were suggested as a useful tool for detecting hydrops, even at early stages.

In this work, we used low-level DPOAEs to assess cochlear function during and after micro-injection of artificial endolymph into the guinea pig’s scala media in order to provide more fun-
damental knowledge regarding the pathophysiological mechanism operational in endolymphatic hydrops.

**Materials and methods**

Experiments were performed in 16 female albino guinea pigs (Harlan, The Netherlands; body weight 350–450 g) with a positive Preyer reflex and a $2f_1 - f_2$-DPOAE of at least 10 dB above the noise floor. Artificial endolymph micro-injections were performed in the right ear of all guinea pigs. The injected volume was 1.1 µl (n=12) with a rate of injection of 90–110 nl/min. In four additional experiments the injected volumes were 4.4 (n=3) and 6.0 µl (n=1) with an injection rate of 530–1060 nl/min. Animal care and use were in accordance with the principles of the declaration of Helsinki and approved by the Groningen animal experiment committee (protocol number 2830).

General anesthesia was induced by intramuscular administration of ketamine/xylazine (60/3.5 mg/kg). Maintenance doses of the anesthetic were administered every hour. Muscle relaxation was obtained with succinylcholine (2.5 mg/kg). The animals were artificially ventilated through a tracheostoma (Columbus Instruments, model 7950) and body temperature was maintained at 38 °C with a heating blanket. The heart rate was monitored with a pair of skin electrodes placed on both sides of the thorax. The animal’s head was kept in a stationary position by means of a steel bolt fixed to the skull with dental cement. Following a retroauricular incision, the bulla and external auditory canal were exposed. Subsequently, the bulla was opened, equalizing middle ear pressure to normal air pressure (Zhang and Abbas, 1997) and exposing the round window.

During the experiment the distortion product otoacoustic emissions were continuously measured using an Etymotic ER-10C DPOAE probe system. The ER-10C probe, consisting of two sound delivery tubes and a microphone port, was directly connected and fixed to a custom made conical connecting tube which was placed air-tight into the external auditory canal of the guinea pig. The two primary frequencies ($f_1, f_2$), evoking the $2f_1 - f_2$ distortion product, were generated by two separate oscillators (HP 4204A). After attenuation, these signals were delivered to the Etymotic ER-10C DPOAE probe system. The two primary frequencies were set at 6 kHz ($f_1$) and 7.5 kHz ($f_2$), $f_2/f_1$-ratio=1.25, with intensities set at respectively, 65 dB SPL ($L_1$) and 55 dB SPL ($L_2$). The sound system was calibrated for the primary frequencies with a 0.1 cm³ coupler connected to a Bruel and Kjær type 2636 half inch microphone. The probe microphone signal was amplified (20 dB), passed a custom made 1.5 kHz high pass filter and was subsequently displayed on a dynamic spectrum analyzer (R9211A Advantest). The noise floor in the 4.5 kHz region averaged to approximately 0 dB SPL. The probe microphone signal was also routed via a SRS dual channel low pass filter (SR640) to a lock-in amplifier (SRS, model SR830 DSP). The lock-in amplifier displayed the amplitude and phase of the DPOAE. It was referenced to an electronic $2f_1 - f_2$-distortion product derived from the original primaries.

The production of this reference electronic $2f_1 - f_2$ signal was performed by two multipliers (AD532JH) in series with subsequent filtering and amplification of the signal.

Through the exposed round window membrane, the tip of a double-barreled micropipette was in-
serted into scala tympani (Fig. 1). After subsequent perforation of the basilar membrane (BM) the micropipette was advanced into scala media. DC potential at the pipette tip was measured to verify its position. The double-barreled micropipettes were drawn from borosilicate glass (1.5/0.84 mm diameter per barrel) and the tips were bevelled (Narishige EG-40). Tip diameters were around 20 µm per barrel, which is a compromise between a low enough flow resistance for fluid injection and a small tip size. One barrel of the pipette was used to measure inner ear pressure and DC potential (WPI 900A micropressure system). Through the other barrel, artificial endolymph (140 mM KCl + 25 mM KHCO₃, (Salt and DeMott, 1997)) was injected with a constant flow rate, by applying a controllable pneumatic pressure with a second WPI 900A micropressure system to the barrel end. The injected volume was measured as the displacement of the fluid meniscus in the pipette, for which the inner diameter is precisely known (0.84 mm). The fluid injection rate was calculated as the total injected volume divided by the total injection time. During an experiment National Instruments LabVIEW was used for data acquisition with a storage rate of 1/s. The recorded output signals were amplitude and phase of the $2f_1-f_2$-DPOAE, DC potential and inner ear pressure. The latter was stored after low pass filtering with a cut-off frequency of 5 Hz. Averaging and smoothing of relevant portions of the obtained recordings and correction of pressure and potential recordings for small linear drift were performed off-line with an appropriate software package.

**Results**

In 10 (out of 12) successfully performed experiments 1.1 µl of artificial endolymph was injected into scala media of the guinea pig at a mean rate of injection of 1.65 nl/s (1.53–1.83). In two experiments there was a suboptimal artificial ventilation with concomitant hypoxia, severely affecting DPOAEs and endocochlear potential (EP). These results were therefore not analysed. In addition-
In four additional experiments a larger and more rapid acute endolymphatic hydrops was created. A representative example of the results is shown in Fig. 5. In this experiment, 4.4 µl of artificial endolymph was injected at a rate of 17.6 nl/s. The immediate rise in inner ear pressure amounted to almost 100 Pa. The EP dropped about 20 mV after a stable period of a few minutes. The $2f_1-f_2$-amplitude dropped from 22 to 10 dB SPL and the $2f_1-f_2$-phase changed almost immediately after the onset of injection to a moment where about 3.0 µl of artificial endolymph was injected (dashed line). At that moment the $2f_1-f_2$-amplitude and -phase dramatically changed, accompanied by a marked change in EP and inner ear pressure. This was interpreted as a “catastrophe” of the endolymphatic system, where the injected volume presumably created a permanent leak somewhere in the membranes bounding the endolymphatic system. In all four experiments the behaviour of the recorded signals after the “catastrophe” showed a great variety, in which no typical patterns could be detected. These results are therefore left out of debate. The “catastrophe” occurred when 3.0–3.5 µl of artificial endolymph was injected.
Figure 2 (a–j): Individual recordings in 10 guinea pigs of $2f_1-f_2$-amplitude (solid line) and $2f_1-f_2$-phase (dashed line) during and after injection of 1.1 µl of artificial endolymph. The grey area depicts the injection period, starting at $t = 100$ s and ending between $t = 700$ s and $t = 820$ s.
Discussion

The total endolymph volume in the inner ear of the guinea pig is 4.7 µl (Shinomori et al., 2001). This means that a volume increase of 1.1 µl amounts to an endolymphatic hydrops of 23%. This degree of hydrops caused a temporary decrease in $2f_1 - f_2$-amplitude of only a few dB. In some guinea pigs (Fig. 2(a) and (d)) almost no DPOAE change was observed, while in other guinea pigs (Fig. 2(c) and (i)) clear changes could be recorded. Apparently, the $2f_1 - f_2$ generating mechanism, which is thought to be related to outer hair cell function is not permanently or substantially influenced by this degree of an acute endolymphatic hydrops. Rask-Andersen et al. (1999) used a similar injection rate of artificial endolymph up to a volume of 1.2 µl to study the function of the endolymphatic sac. After injection they immediately terminated the guinea pig for morphological study of the endolymphatic sac. They found a dynamic relationship between active secretion and enzymatic degradation of a lumen-expanding homogeneous substance that was intimately related to the intraluminal macrophages. These authors suggest that this homogeneous substance could play an important role in the regulation of endolymph volume in the endolymphatic system. However, explanation of the observed recovery of the $2f_1 - f_2$-amplitude during injection by a counteracting absorption of endolymph is contradicted by the behaviour of inner ear pressure during injection, as shown in Fig. 4. When inner ear pressure is suddenly changed by injection of artificial endolymph, pressure adjusts to a new equilibrium value within seconds. In the new situation fluid inflow equalizes fluid outflow through the cochlear aqueduct. If outflow (either endolymph or perilymph) would increase during this period, a decrease of pressure should be observed, which

Figure 3: Averaged changes (n=10) in $2f_1 - f_2$-amplitude (upper panel) and $2f_1 - f_2$-phase (lower panel) during and after injection of 1.1 µl of artificial endolymph. The grey area depicts the injection period, starting at $t = 100$ s and ending at various times for individual experiments between $t = 700$ s and $t = 820$ s. Standard error bars for the changes with respect to the pre-injection values are shown at $t = 480$ s, when the $2f_1 - f_2$-amplitude reaches a minimum during injection, and $t = 1000$ s.
Figure 4: Typical results from an experiment in which 1.1 µl of artificial endolymph was injected in the guinea pig’s scala media. The grey area depicts the injection period. Panel 1 (upper panel): calculated volume of endolymph in endolymphatic space; 1.1 µl is added to the normal endolymph volume of 4.7 µl. Panel 2: pressure profile measured in scala media. Panel 3: Endocochlear potential (EP) profile. Panel 4: 2f₁-f₂-amplitude profile. Panel 5 (lower panel): 2f₁-f₂-phase profile. The lower two panels correspond to Fig. 2(b).
Figure 5: Typical results from an experiment in which 4.4 µl of artificial endolymph was injected in the guinea pig’s scala media. The grey area depicts the injection period. Panel 1 (upper panel): calculated volume of endolymph in endolymphatic space, 4.4 µl is added to the normal endolymph volume of 4.7 µl. Panel 2: pressure profile measured in scala media. Panel 3: Endocochlear potential (EP) profile. Panel 4: $2f_1 - f_2$-amplitude profile. Panel 5 (lower panel): $2f_1 - f_2$-phase profile. The dashed line corresponds with the “catastrophe”, after which the endolymphatic system is damaged (see main text).
is not the case. From the recorded pressure increase during injection the flow resistance of the cochlear aqueduct can be calculated by dividing the pressure increase by the rate of injection. This yielded a mean cochlear aqueduct flow resistance of 14.3 Pa s/nl (Table 1), which is in accordance with the results of Wit et al. (1999).

To document the limits of the endolymphatic system when coping with an acute induced hydrops, volumes of 4.4 and 6.0 µl of artificial endolymph were injected with a higher rate, yielding an acute hydrops of 94% and 128%. After 3.0–3.5 µl (64–74%) was injected a “catastrophe” occurred, similar as in the injection experiments of Wit et al. (2000). Probably a permanent leak was created somewhere in the endolymphatic system causing a substantial drop in 2f₁-f₂-amplitude, indicating a marked disturbance in cochlear function.

As far as we know from available literature, there are only two studies of cochlear function in an acute induced endolymphatic hydrops model monitored by means of electrocochleography. Jin et al. (1990) found a rise of the summating potential (SP), a decrease of action potentials (AP) amplitude and an increase in SP/AP ratio, when artificial endolymph was injected. Sirjani et al. (2003) injected volumes up to 1.2 µl in a 15 min period. EP and CM (cochlear microphonics) amplitude at 8 kHz were almost unaffected, while SP, CM distortion and operating point showed substantial changes, of which the direction and magnitude varied in each guinea pig. These observations can be explained by a change of the operating point of the transducer channels in the apical membrane of the OHC (van Emst et al., 1997), as a consequence of a permanent displacement of the BM.

In contrast to the acute model, the chronic endolymphatic hydrops model has been extensively studied using DPOAEs. By obliterating the endolymphatic sac Horner et al. (1991), Magliulo et al. (1996) and Okubo et al. (1997) induced a chronic hydropic ear. They reported the DPOAE to be a sensitive method for detecting the presence of hydrops. Magliulo et al. (1996) found a close relationship of the functional effects of hydrops on the results of compound action potentials (CAPs) and DPOAEs. They argued for DPOAE testing to monitor the progression of cochlear dysfunction in a hydropic ear. Horner (1991) found reduced DPOAEs in hydropic cochleas when the primary tones lay within the pathological part of the CAP audiogram. They proposed that low-frequency CAPs sensitivity loss in early hydrops is of hair cell origin. Their data support the contention that DPOAEs can provide information on the functioning of outer hair cells. The amount of DPOAE change in our acute hydrops model is less than the changes reported in the chronic hydrops model. An explanation for this might be found in the complicated pathology in later stages of chronic experimental hydrops. Biochemical modifications to the composition of the endolymph and substantial loss of hair cells (Horner, 1991) are secondary effects likely to be responsible for more severe changes in cochlear functioning.

It is widely accepted that the outer hair cells are responsible for the exquisite sensitivity, frequency selectivity and dynamic range of the cochlea. The OHCs are part of a mechanical feedback system involving the BM and tectorial membrane. Displacement of the BM results in a relative motion between the tectorial membrane and the reticular lamina, which causes deflection of the stereocilia with modulation of the open probability of the OHCs transduction channels. The resulting current is also fed back into the motion of the BM. Nowadays, it is thought that DPOAEs disappear when outer hair cells stop enhancing the BM motion, so that it loses its tuning and sensitivity.
A static displacement of the BM has been put forward as an explanation for low frequency hearing loss in Menière’s disease (Tonndorf, 1957, 1976). In this explanation a pressure difference, which is dependent on the mechanical compliance of the membranous labyrinth, displaces the BM from its resting position, thereby impairing cochlear function. According to Böhmer (1993), the presence of a positive endolymphatic–perilymphatic pressure gradient in chronic experimental endolymphatic hydrops was indeed partially correlated to deterioration of auditory thresholds.

Considering these findings, it is of interest to estimate the BM displacement at the site of the $2f_1-f_2$-location for the acute induced endolymphatic hydrops.

The volume compliance of a part of the BM with length $\Delta x$, at position $x$ is given by $C(x)\Delta x$. By integrating this expression over the length $L$ of the BM, the total compliance of the BM is obtained:

$$C_{BM} = \int_{0}^{L} C(x)\,dx.$$  \hfill (1)

It is reasonable to assume that $C(x)$ is an exponential function of $x$ (de Boer, 1996):

$$C_x = C_0 e^{\alpha x}$$  \hfill (2)

in which $C_0$ is the volume compliance for $x=0$.

Combining Eqs. (1) and (2) gives

$$C = \frac{C_0}{\alpha} [e^{\alpha L} - 1]$$  \hfill (3)

Décory et al. (1990) provided a value for the compliance of the entire BM of the guinea pig. This value for $C_{BM}$ is given as $9 \times 10^{-14}$ m$^3$/N. Furthermore, $C_0 = 4.4 \times 10^{-14}$ m$^3$/N, as can be derived (see Appendix A) from $S_p=1.25$ N/m; which is a measured value for the point stiffness at the guinea pig BM base (Gummer, 1981; formula (28)). This value is in the range of $1.9–6.4 \times 10^{-14}$ m$^3$/N, provided by Ruggero (1990, Table IV, 2001). Substituting these values for $C_{BM}$ and $C_0$ in Eq. (3) gives $\alpha = 0.36$ mm$^{-1}$, for $L = 18.5$ mm (Greenwood, 1990). With this value for $\alpha$ Eq. (2) can be written as: $C(x) = 4.4 \times 10^{-14} e^{0.36x}$ m$^3$/N ($x$ in mm).

According to this relation, $C(x)$ changes with a factor $e^{0.36} \approx 800$ from base to apex. The progressive decrease in BM stiffness from base to apex explains the cochlear frequency map. Extrapolating from measurements on dissected cadaver cochleas of several species, von Békésy (1960) estimated a stiffness ratio of $\approx 10,000$. However, in excised gerbil cochleas Naidu and Mountain (1998) found the base-to-apex point stiffness ratio to be 100, which is two orders of magnitude
smaller. These authors challenge the theory in which the cochlear frequency map solely depends on the progressive decrease in partition stiffness from base to apex. Our compliance ratio estimation for the guinea pig (800) supports their opinion because a factor of nearly 10,000 is needed for the cochlear frequency range to depend on BM stiffness only (Robles and Ruggero, 2001). Avan et al. (1998) provided an estimation for the generation place of the $2f_1-f_2$-DPOAE. It is close to the place tuned to $f_1$ for stimulus levels lower than 70 dB SPL. In our study $f_2$ was 7.5 kHz. Using the relation $f = 0.35(10^{0.1135x} - 0.85)$ between frequency and location on the BM as given by Greenwood (1990), (where $x$ is the distance from helicotrema in mm), the $f_2$-location is found at 6.6 mm from stapes. Substituting the values for $C_0$, $\alpha$ and $x$ in Eq. (2) renders a volume compliance of the BM at the $2f_1-f_2$-location of $4.74 \times 10^{-7}$ mm$^2$/Pa. The injection of 1.1 µl of artificial endolymph increases the pressure difference between endolymph and perilymph with $\approx 3.5$ Pa (Wit et al., 2000 (Fig. 10)). The calculated volume compliance, taking a parabolic shape for the cross section of the BM and a width of 130 µm (Lewis et al., 1985), gives a BM-displacement of 19 nm at the $2f_1-f_2$-location. This value is within the functional range of BM displacements, which is between approximately -10 and +40 nm and corresponds with a full dynamic range of the OHC stereocilia of $\approx 2^\circ$ angular displacement (Dallos, 2003). So injection of artificial endolymph leads to a small deflection of the hair cell stereocilia and as a consequence a change in hair cell conductance, which could explain the observed $2f_1-f_2$ characteristics. It might be that after some time during injection the position of the BM is returning to its pre-injection position through some (unknown) mechanism. In this case the displacement of the BM at the $2f_1-f_2$ generation site is smaller than 19 nm.

Withnell et al. (2003) found that the guinea pig DPOAE is a composite of two sine waves arising from different mechanisms, one which is non-linear distortion, the other being linear coherent reflection. In an attempt to explain the typical course of the $2f_1-f_2$-amplitude with its recovery starting within the injection period, we analysed a two sine wave summation model (see Appendix B). This model can explain the shape of the $2f_1-f_2$-amplitude curve, if it is assumed that one sine wave decreases monotonically, while the other remains constant. It can even exactly fit the shape of the amplitude curve by a proper choice of the amplitude decrease of the first sine wave. However, this fit requires a phase shift of $78^\circ$ during amplitude change (Appendix B, Eq. (B.5)). Such a large phase shift was observed in none of the animals (Fig. 2).

The levels of primaries were carefully chosen with respect to the two-component model of Mills (1997), in which he describes a level-dependent sensitivity to impairment of OHCs. DPOAEs can be evoked by high-level or low-level primaries. The high level DPOAEs can persist despite cochlear pathology, whereas the low level DPOAEs systematically disappear whenever the cochlear impairment is large enough. The DPOAEs generated by the low-level primaries that we used proved to be sensitive to changes in cochlear physiology. In pilot experiments guinea pigs (n=2) were made temporarily hypoxic by blocking of artificial ventilation for a brief period of time. The resulting substantial decrease and recovery of the measured DPOAEs attested to their physiological origin.

Reduction of the EP severely decreases low-level DPOAEs (Mills et al., 1993). Therefore, artificial endolymph with the same electrolyte composition as natural endolymph was used (Salt and DeMott, 1997). Also, a very low injection rate was chosen to minimize possible mechanical
effects. Kakigi and Takeda (1998) investigated the effect of artificial endolymph injection on the EP. They observed a slight increase of the +EP during injection (max. 500 nl/min) with a return to initial values within minutes after the end of injection. The same phenomenon was observed in this study.

The present study clearly demonstrates modest effects on DPOAE-amplitude and -phase during and after induction of an acute endolymphatic hydrops, which do not merely follow change of inner ear pressure. Therefore, the disturbance of endolymph volume itself is likely to be responsible for the observed effects. By simultaneously measuring DPOAE-changes and changes in cochlear microphonics (CM) distortion, we expect to obtain more insight in the changes that occur in the inner ear during an acute hydrops. The measurement of CM-distortion, in particular of the $f_2-f_1$ and $2f_1$-amplitudes, may provide information about the cochlear transducer function (Bian et al., 2002) and changes in its operating point during injection of artificial endolymph.

Appendix A

Gummer et al. (1981) measured the point stiffness of the guinea pig’s BM by displacing it with a probe with a diameter $w = 25 \mu m$. The work done by the force $F$ exerted by the probe is $W = F \cdot d$, in which $d$ is the displacement of the BM at the position of the probe tip. As $F = S \cdot d$ ($S =$ membrane point stiffness), this amount of work is also given by

$$W = S \cdot d^2 \quad \text{(A.1)}$$

If a pressure $p$ causes a displacement $y(x)$ of a basilar membrane ribbon with width $w$, the work done by $p$ is given by (Fig. 6).

$$W = \int_{-w/2}^{w/2} py(x) \, dx$$

This gives

$$W = pwA \quad \text{(A.2)}$$

in which $A$ is the area under the curve in Fig. 6(a). We assume that the amount of work given by (A.1) and (A.2) are equal. Hence:

$$S = \frac{pwA}{d^2} \quad \text{(A.3)}$$
The volume compliance of the displaced BM ribbon is given by

\[ C_x = \frac{A}{p} \]  \hspace{1cm} (A.4)

So, from (A.3) and (A.4) we get:

\[ C_x = \frac{w}{S} \left( \frac{A}{d} \right)^2. \]  \hspace{1cm} (A.5)

If we assume that \( y(x) \) has a parabolic shape \( A = 2/3 \, ad \), and (A.5) can be written as:

\[ C_x = \frac{4 \, wa^2}{9 \, S}. \]  \hspace{1cm} (A.6)

For the basal part of the guinea pig cochlea \( a = 70 \, \mu m \) (Lewis et al., 1985). This gives \( C_0 = 5.44 \times 10^{-14}/S_0 \, \text{m}^4/\text{N} \). With \( S_0 = 1.25 \, \text{N/m} \), we get \( C_0 = 4.4 \times 10^{-14} \, \text{m}^4/\text{N} \).

**Appendix B**

If two sine waves \( a_1 \sin \omega t \) and and \( a_2 \sin (\omega t + \varphi) \) are added, the amplitude of the resulting sine wave is given by:

\[ A = \sqrt{a_1^2 + a_2^2 + 2a_1a_2\cos\varphi}, \]  \hspace{1cm} (B.1)

and the phase angle (with respect to \( a_1 \sin \omega t \)) by:

\[ \cos \psi = \frac{a_1 + a_2 \cos \varphi}{A}. \]  \hspace{1cm} (B.2)
If we assume $\varphi$ to remain constant, changes of $A$ and $\psi$ depend on the behaviour of $a_1$ and $a_2$ as a function of time. If $a_1$ decreases monotonically and $a_2$ remains constant, $A$ passes through a minimum at the moment that

$$a_1 = -a_2 \cos \varphi.$$  \hspace{1cm} (B.3)

This condition can only be fulfilled if $\cos \varphi < 0$. The minimum has the value:

$$A_{\text{min}} = a_2 \sin \varphi.$$  \hspace{1cm} (B.4)

The total phase shift of the resulting sine wave is given by:

$$\alpha = \pi - \left(\arcsin \frac{A_{\text{min}}}{A_f} + \arcsin \frac{A_{\text{min}}}{A_0}\right)$$  \hspace{1cm} (B.5)

in which $A_0$ and $A_f$ are the initial value and final value of $A$, respectively.

References


