Ultrasound-Mediated Transdermal Drug Delivery  
Mechanisms and Applications  

By  
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ABSTRACT

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Transdermal drug delivery (TDD) offers several advantages over the traditional drug delivery methods such as oral delivery and injection. When compared to oral delivery, TDD avoids gastrointestinal drug metabolism, reduces first-pass effects, and provides sustained release of drugs for up to 7 days. When compared to injections, TDD eliminates the associated pain and the possibility of infection. Transdermal drug delivery, however, suffers from the severe limitation that the permeability of the skin to drugs is very low, thus limiting its clinical applications. The low skin permeability is due to the stratum corneum (SC), the outermost layer of the skin.

Application of ultrasound has been suggested to enhance transdermal transport of drugs, a phenomenon referred to as phonophoresis or sonophoresis. Although significant attention has been devoted to the investigation of sonophoresis in recent years, sonophoresis suffers from several limitations including: i) most attempts of sonophoresis have been performed using ultrasound in a narrow parameter range referred to as the therapeutic range (frequency- 1-3 MHz, intensity- 0-2 W/cm²), ii) the enhancement is restricted to low-molecular weight compounds, iii) a typical enhancement is less that 10-fold, and iv) the mechanisms of sonophoresis are not clearly understood. These limitations of sonophoresis motivate this thesis.

Mechanistic studies of sonophoresis were performed to evaluate the roles played by various ultrasound-related phenomena, including cavitation, thermal effects, generation of convective velocities, and mechanical effects, in sonophoresis. The experimental findings suggest that among the ultrasound-related phenomena evaluated, cavitation plays the dominant role in therapeutic sonophoresis. Furthermore, confocal microscopy results indicate that cavitation occurs in the keratinocytes of the SC upon ultrasound exposure. It was hypothesized that oscillations of the cavitation bubbles induce disorder in the SC lipid bilayers thereby enhancing transdermal transport. A theoretical model was developed in order to predict the effect of ultrasound on the transdermal transport of drugs. The model predicts that sonophoretic enhancement under therapeutic conditions depends most directly on the passive permeant diffusion coefficient, rather than on the permeability coefficient through the skin. Specifically, permeants passively diffusing through the skin at a relatively slow rate are expected to be preferentially enhanced by therapeutic ultrasound. The experimentally measured sonophoretic transdermal transport enhancements for seven permeants, including estradiol, testosterone, progesterone, corticosterone, benzene, butanol, and caffeine, agree quantitatively with the model predictions.
Since cavitation effects vary inversely with ultrasound frequency, it was hypothesized that ultrasound at frequencies lower than those corresponding to therapeutic ultrasound should be more effective in enhancing transdermal drug transport as compared to therapeutic ultrasound. Indeed, application of ultrasound at 20 KHz induced transdermal transport enhancements of up to 1000 times higher than those induced by therapeutic ultrasound. In vitro experiments performed with seven permeants, including estradiol, aldosterone, corticosterone, sucrose, water, butanol, and salicylic acid, show that application of low-frequency ultrasound (20 KHz, 125 mW/cm², 100 msec pulses applied every second) enhances transdermal transport of a wide variety of drugs by a factor in the range of 3 to 5000. In vivo experiments performed using salicylic acid as a model drug and hairless rats as an animal model indicate that low-frequency ultrasound can also enhance transdermal transport across living skin by a factor of about 300. A preliminary safety analysis indicates that low-frequency ultrasound does not induce a long-term loss of the barrier properties of skin (in vitro). In addition, histological studies of the hairless rat skin exposed to ultrasound (in vivo) suggest that ultrasound exposure under the conditions used for low-frequency sonophoresis does not induce any damage to living skin or underlying tissues.

A mechanistic explanation for the observed enhancement of transdermal transport by low-frequency sonophoresis is provided. It is hypothesized that application of low-frequency ultrasound results in disordering of the SC lipid bilayers due to cavitation. Furthermore, a significant amount of water from the keratinocytes may penetrate into the disordered lipid domains resulting in the formation of aqueous channels across the SC through which transdermal transport may occur. Support for this hypothesis is provided using experimental and theoretical analyses of low-frequency sonophoresis.

Low-frequency ultrasound was also found to increase the permeability of human skin to high-molecular weight proteins by several orders of magnitude. It was possible to deliver therapeutic doses of proteins such as insulin, γ-interferon, and erythropoietin across human skin.

The efficacy of low-frequency ultrasound in enhancing the transdermal transport of high-molecular weight proteins, for example, insulin, as well as of low-molecular weight drugs such as salicylates, makes it a potential non-invasive substitute for injections. With further research, one might envision small pocket-size sonicators carried by the patients and used to "inject" drugs whenever required. In addition, these devices could be potentially combined with sensors that can monitor drug concentrations in the blood to formulate a self-controlled drug (insulin, for example) delivery method that can potentially eliminate the attention required by the patient.

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1. Introduction

1.1 Transdermal Drug Delivery: Transdermal drug delivery (TDD) offers an alternative to oral delivery and injections. When compared to oral delivery, TDD avoids gastro-intestinal drug metabolism, reduces first-pass effects, and provides sustained release of drugs for up to 7 days [1]. When compared to injections, TDD eliminates the associated pain and the possibility of infection. Despite these advantages, applications of TDD are currently restricted to only seven drugs (estradiol, testosterone, nicotine, fentanyl, clonidine, nitroglycerine, and scopolamine) because of the skin’s low permeability [1]. The enormous barrier properties of the skin are attributed to the stratum corneum (SC), the outermost layer of the skin (Figure 1a). The SC is about 15 μm thick and consists of keratinocytes, which are disk-like dead cells filled with cross-linked keratin fibers that are surrounded by lipid bilayers (Figure 1b).

![Figure 1a](image1.png)

**Figure 1a**

**Figure 1**- Schematic representation of the skin structure including various transdermal transport pathways. The major transport pathway comprises the intercellular lipid bilayers. Figure 1a shows three principal layers of the skin, that is, the stratum corneum, the epidermis, and the dermis. A section of the stratum corneum is blown up in Figure 1b.

![Figure 1b](image2.png)

**Figure 1b**

Each keratinocyte is about 23 μm in diameter and about 1 μm in thickness. Two layers of keratinocytes are separated by a 50 nm thick layer of lipid bilayers. Each bilayer is about 5 nm thick, suggesting that there are about 10 lipid bilayers between two layers of keratinocytes. The transdermal transport of drugs occurs through the intercellular lipid bilayers whose ordered
structure confers a low permeability to the skin [2]. Although hair follicles and sweat ducts may potentially contribute to transdermal transport, they occupy a small surface area of the skin (~ 0.01%) and therefore may not contribute significantly to passive transdermal transport. More information on permeation pathways of transdermal transport can be found in Refs. [2, 3].

A variety of approaches have been suggested to enhance transdermal drug transport. These include: i) use of chemicals to either modify the skin structure or to increase the drug concentration in the transdermal patch used to deliver drugs [4, 5], ii) application of electric fields to create transient transport pathways (electroporation) [6, 7] or to increase the mobility of charged drugs through the skin (iontophoresis) [8], and iii) application of ultrasound (phonophoresis and sonophoresis) [9].

1.2. Phonophoresis: Phonophoresis refers to the use of ultrasound for enhancing local transdermal delivery of drugs. The first report on phonophoresis dates back to 1954 when Fellinger et al. [10] used ultrasound to enhance local delivery of hydrocortisone across pig skin. Since then, phonophoresis has been shown to enhance transdermal transport of more than 15 drugs [9, 11-53]. During the time period between 1950 and 1970 the transdermal route of drug administration was adopted for topical (local) rather than systemic delivery of drugs. Hence, most of the ultrasound-enhanced transdermal drug delivery experiments conducted during this period were aimed at topical delivery of drugs. A summary of literature reports on phonophoresis is provided in Chapter 4. The most commonly used drugs for phonophoresis include steroidal anti-inflammatory drugs such as hydrocortisone and dexamethasone, non-steroidal anti-inflammatory drugs such as indomethacin and salicylate, and local anesthetic agents such as lidocaine. The most commonly used technique of phonophoresis was to apply the drug in the form of an ointment on the skin, followed by application of ultrasound keeping the transducer (part of sonicator that generates ultrasound. See Chapter 2 for details) in contact with the ointment. In some cases, the ultrasound transducer was moved in circular patterns to avoid a continuous exposure of a certain part of the skin to ultrasound. To the surprise of most investigators, the observed ultrasound enhancement of transdermal transport varied from drug to drug. For example, in the case of hydrocortisone, a significant enhancement was observed in many studies, however, most of the attempts to enhance transdermal transport of lidocaine and salicylates were less successful. This variation of the efficacy of phonophoresis from drug to drug raised a controversy in the literature regarding the applicability of phonophoresis as a transdermal transport enhancer [33].

Over the last 20 years, the transdermal route of drug delivery has been considered as a means for systemic drug administration. In this time period, application of ultrasound has been attempted to enhance systemic transdermal drug delivery. This method is referred to as
sonophoresis. The first experiments documenting the use of sonophoresis were reported by Kost et al. in 1986 who showed [31] that 3-5 minutes of therapeutic ultrasound exposure (1 MHz, 1.5 W/cm²) increased transdermal permeation of mannitol and physostigmine across hairless rat skin in vivo by up to 15-fold. It was also reported that the lag time typically associated with transdermal drug delivery was nearly-completely eliminated after exposure to therapeutic ultrasound [36]. Following this study, the enhancement of systemic drug delivery by sonophoresis was reported by several other investigators [15, 16, 40], although the sonophoretic enhancement was limited to low-molecular weight drugs and was typically less than 10-fold. In many cases, no enhancement of transdermal drug transport was observed upon application of ultrasound [12-14, 43, 46, 52].

It is noteworthy that the most commonly used condition for sonophoresis corresponds to therapeutic ultrasound (frequency in the range of 1-3 MHz). Specifically, out of all the literature reports on sonophoresis or phonophoresis, over 85% involve use of therapeutic ultrasound, although frequencies above 3 MHz and below 1 MHz have also been used by a few investigators [15, 16, 49, 54].

Significant attention has also been devoted to elucidate the mechanisms of sonophoresis (for the sake of simplicity, the words sonophoresis and phonophoresis are used here onwards indistinguishably to indicate ultrasound-enhanced transdermal drug transport). Three mechanisms have been considered to play important role in sonophoresis; i) thermal effects, ii) convection (acoustic streaming), and iii) cavitation effects. A brief description of these effects is provided below.

a. Thermal Effects. Absorption of ultrasound results in a temperature increase of the medium. Materials which possess higher ultrasound absorption coefficients, such as bones, experience severe thermal effects as compared to tissues such as muscle which have a lower absorption coefficient, α. α-values for several biological tissues can be found in Ref.[55, 56] (see also Table 1 in Chapter 2). The absorption coefficient of a medium increases proportionally with the ultrasound frequency, indicating that thermal effects associated with ultrasound are proportional to the ultrasound frequency. The increase in the temperature of a medium upon ultrasound exposure at a given frequency varies proportionally with the ultrasound intensity and exposure time. The thermal effects can be substantially decreased by a pulsed application of ultrasound. For a detailed discussion of the thermal effects of ultrasound, see Ref. [55].

b. Acoustic Streaming. Acoustic streaming, by definition, is the development of time independent large fluid velocities in a medium under the influence of an ultrasound wave. The primary cause of acoustic streaming are the reflections and other distortions of the wave propagation.
Oscillations of cavitation bubbles (see below) may also contribute to acoustic streaming. The shear stresses developed by streaming velocities may affect the neighboring structures [57].

c. Cavitation Effects. Cavitation refers to the formation of gaseous cavities in a medium upon ultrasound exposure. The primary cause of cavitation is the ultrasound-induced pressure variations in the medium. Cavitation involves either rapid growth and collapse of a bubble (transient cavitation), or the slow oscillatory motion of a bubble in an ultrasound field (stable cavitation). Cavitation affects tissues in several ways. Specifically, collapse of cavitation bubbles releases a shock wave which can cause structural alterations in its surroundings. Biological tissues contain numerous air pockets trapped in the fibrous structures which act as nuclei for cavitation upon ultrasound exposure. Accordingly, a significant cavitation activity is known to occur in biological tissues upon ultrasound exposure [55]. Cavitational effects vary inversely with ultrasound frequency and directly with ultrasound intensity [55].

Several investigators have speculated about the roles played by the above mentioned effects in sonophoresis. Mortimer et al. [41] performed sonophoresis of oxygen across frog skin in vitro. They found that the sonophoretic enhancement of transdermal oxygen transport depends on ultrasound intensity, rather than on pressure amplitude. Based on this observation, they hypothesized that cavitation cannot be responsible for sonophoresis, since cavitational effects are known to be proportional to ultrasound pressure rather than intensity. They hypothesized that the observed enhancement occurs due to acoustic streaming in the solution around the skin [41]. Levy et al. [36] performed an in vitro investigation of the roles played by thermal effects, cavitation, and mixing in the sonophoretic enhancement of urea transport across polymer membranes. They found that the observed enhancement cannot be explained by thermal effects or mixing. In an attempt to elucidate the role played by cavitation, they performed sonophoresis experiments using degassed buffer solutions and found that the degassing procedure reduced the sonophoretic enhancement of urea permeation by 2-fold. Since degassing a solution decreases the cavitation activity in the solution, they hypothesized that cavitation may play a role in sonophoresis. Simmonin et al. [58] hypothesized that cavitation occurs in the follicles of the skin upon ultrasound exposure, and enhances transdermal permeation by generating convective velocities through follicles. However, no evidence was presented to support this hypothesis. Similar conclusions were reached by Tachibana et. al. [49, 54]. Bommanan et al. [15] performed sonophoresis of lanthanum tracers across hairless mice skin at an ultrasound frequency of 16 MHz. They reported that the tracer was patchily distributed within the intercellular lipid bilayers of the SC. They hypothesized that the patchy distribution of the lanthanum tracer revealed in the micrographs corresponds to the location of oscillating air pockets in the SC. Similar observations were reported by Menon et al.
[39], however, precise mechanisms by which cavitation enhances transdermal transport were not presented. Therefore, despite multiple past investigations, the mechanism responsible for sonophoresis remains unclear.

In summary, a review of the sonophoresis literature reveals two critical issues. First, there appears to be a need for a better selection of ultrasound parameters which may improve the efficiency of sonophoresis compared to that of therapeutic sonophoresis and second, there appears to be a need for a better understanding of the mechanisms responsible for sonophoresis which could potentially lead to a rational selection of ultrasound parameters for better efficacy as well as to the development of mathematical models to predict sonophoretic enhancement. Accordingly, the broad objectives of this thesis are to develop a better fundamental understanding of sonophoresis and to utilize this understanding to improve the efficacy of sonophoresis.

1.3. Objectives: The specific objectives of this thesis are:

1. To understand the transport pathways and mechanisms of sonophoresis: Specifically,
   i) To understand which of the various ultrasound-related phenomena, including thermal effects, convective transport, mechanical effects, and cavitation, is responsible for sonophoresis.
   ii) To understand how the above mentioned phenomena enhance transdermal transport
   iii) To understand, at a fundamental level, the limitations of therapeutic ultrasound in enhancing transdermal transport.
   iv) To develop mathematical models to predict the effect of ultrasound on transdermal transport under various conditions.

2. To utilize the mechanistic understanding gained in objective 1 to improve the efficacy of sonophoresis: Specifically,
   i) To select proper ultrasound parameters to selectively enhance the ultrasound-related phenomenon responsible for sonophoresis.
   ii) To assess whether ultrasound under the new selected conditions enhances transdermal transport of a broad variety of drugs including proteins.
   iii) To evaluate mechanisms of sonophoresis under the new selected conditions.
   iv) To develop mathematical models to predict sonophoretic transport under the new conditions.

3. To evaluate safety issues related to sonophoresis at a preliminary level: Specifically,
   i) To evaluate the biological effects of low-frequency ultrasound on the skin tissue.
   ii) To evaluate the recovery of the barrier properties of the skin after sonophoresis.
2. Overview of Ultrasound

Ultrasound is a sound wave possessing frequencies above 20 KHz [59, 60]. Ultrasound waves are characterized by two main parameters: frequency and amplitude. The amplitude of ultrasound waves can be represented in terms of the peak wave pressure, $P$, (in units of Pascals) or in terms of the intensity, $I$, (in units of $W/cm^2$). $P$ and $I$ are related by the equation $I = P^2 / 2\rho c$, where $\rho$ is the water density (1000 kg/m$^3$), and $c$ is the velocity of ultrasound in water (1500 m/s). Ultrasound can be applied either continuously or in a pulsed manner. In the latter case, an additional parameter, the duty cycle, is required to characterize the ultrasound application. Duty cycle is the fraction of time during which ultrasound is ON. Thus, ultrasound application for sonophoresis may be completely characterized by four main parameters, frequency, intensity, duty cycle, and exposure time. Ultrasound is generated using a device referred to as a sonicator which consists of an electrical signal generator capable of generating an electrical AC signal at the desired frequency and amplitude. This signal is applied across a piezo-electric crystal (also known as the transducer) to generate ultrasound. The thickness of the piezo-electric crystal is selected so that it resonates at the operating frequency. Sonicators operating at various frequencies in the range of 20 KHz to 3 MHz are available commercially and can be used for sonophoresis. If a sonicator operating at the desired frequency is not available commercially, it is possible to assemble one using commercially available signal generators, amplifiers, and transducers [16]. For a detailed description of methods for sonicator assembly, see Ref. [16].

Ultrasound under different conditions has been used in medicine for various purposes. The main current clinical applications of ultrasound include:

i) **High frequency ultrasound** ($>3$ MHz) for diagnostic applications [56]. A few diagnostics applications of ultrasound include: a) imaging of babies, b) determination of skin thickness, and c) determination of blockages in blood circulation. The physical principle underlying ultrasonic imaging includes the measurement of the time lag between the incident and reflected ultrasound wave from various parts of the tissue to be imaged. The resolution of the image is directly related to the ultrasound wavelength. Accordingly, low-wavelength or high-frequency ultrasound is preferred for diagnostic applications. However, as will be described later, the ultrasound absorption coefficient of a material varies directly with ultrasound frequency, thus
prohibiting the use of excessively high ultrasound frequencies for diagnostics. The typical frequency range used for diagnostics application is 3-10 MHz (pulsed application).

ii) **Low-frequency ultrasound** ($f\sim 20-40$ KHz) for dental cleaning [61, 62]. The cleaning effect of ultrasound is attributed to cavitation and streaming which may remove plaque from the teeth. A typical low-frequency ultrasound dental cleaning device includes a signal generator (20-40 KHz) connected to a sickle-shaped transducer which a dentist can use to clean the teeth. Other possible clinical applications of low-frequency ultrasound ($f\sim 20$ KHz) include liposuction and angioplasty. In the former case, low-frequency ultrasound is applied to the desired region of the body in order to melt the local fat tissue, which is then sucked out using needles. In ultrasound based angioplasty, small ultrasound transducers are inserted inside the blood vessels for cleaning as well as for opening of closed blood vessels. Clinical trials of ultrasound liposuction as well as angioplasty are being carried out by Misonix Inc., Farmingdale, NY.

iii) **Therapeutic ultrasound** ($1$ MHz<$f< 3$ MHz) for sonophoresis [32, 33, 48]. A review on use of ultrasound for sonophoresis was provided in Chapter 1. For sonophoretic delivery, the desired drug is dissolved in a solvent and applied on the skin. Ultrasound is applied by contacting the ultrasound transducer with the skin through a coupling medium to ensure proper contact between the transducer and the skin (Figures 2A and 2B).

![Figure 2A](image)

![Figure 2B](image)

Figure 2- Experimental arrangement for in vitro (2A) and in vivo (2B) sonophoresis experiments.

The coupling medium can be the same as the solvent used to dissolve the drug, or it can be a commercially available ultrasound coupling gel (for example, Aquaasonic, Polar, N. J.). The coupling medium should result in proper transmission of ultrasound from the transducer to
the skin. The transmittive properties of a medium are indicated by its acoustic impedance, \( Z \). A coupling medium is appropriate for sonophoresis if its acoustic impedance, \( Z \), is comparable to that of the skin (\( 1.6 \times 10^6 \) kg/m\(^2\)/s). \( Z \)-values for various materials can be found in Refs. [59, 60] (see also Table 1). As an example, water has a \( Z \)-value of \( 1 \times 10^6 \) kg/m\(^2\)/s and is therefore a reasonable coupling agent.

Every medium absorbs ultrasound to a certain extent, and its ability to do so is indicated by the absorption coefficient, \( \alpha \). The extent of absorption is given by the following equation:

\[
f(\tau) = 1 - \exp(-\alpha \tau)
\]

where \( f(\tau) \) is the fraction of ultrasound intensity absorbed as the ultrasound beam propagates through a medium having absorption coefficient \( \alpha \) and thickness \( \tau \). Estimated values of \( \alpha \) for various materials at various ultrasound frequencies may be found in Refs. [59, 60]. In the case of water, for example, the \( \alpha \) value is 0.0006 at an ultrasound frequency of 1 MHz, suggesting that a 1 cm thick column of water absorbs less than 0.1% of ultrasound (1 MHz) intensity. In other words, water is a reasonable coupling medium. Accordingly, water was the coupling medium used in the in vitro as well as in vivo experiments reported in this thesis. \( Z \) and \( \alpha \) values of several biological media are listed in Table 1.

<table>
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<tr>
<th>Biological Medium</th>
<th>Acoustic Impedance, ( Z ) (Kg/m(^2)/s)</th>
<th>Absorption Coefficient ( \alpha ) at 1 MHz (cm(^{-1}))</th>
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<tr>
<td>Air</td>
<td>0.0004 ( \times ) ( 10^6 )</td>
<td>2.76</td>
</tr>
<tr>
<td>Blood</td>
<td>( 1.6 \times 10^6 )</td>
<td>0.028</td>
</tr>
<tr>
<td>Bone</td>
<td>( 6.3 \times 10^6 )</td>
<td>3.22</td>
</tr>
<tr>
<td>Fatty Tissue</td>
<td>( 1.54 \times 10^6 )</td>
<td>0.14</td>
</tr>
<tr>
<td>Muscle</td>
<td>( 1.6 \times 10^6 )</td>
<td>0.76</td>
</tr>
<tr>
<td>Skin</td>
<td>( 1.6 \times 10^6 )</td>
<td>0.62</td>
</tr>
<tr>
<td>Water</td>
<td>( 1.5 \times 10^6 )</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

Table 1- Acoustic impedances and absorption coefficients of several biological media.

In general, ultrasound is reflected at the boundary between two media possessing different acoustic impedances. 99.99% of ultrasound is reflected at the air-water boundary when an ultrasound beam impinges on it from either side. Hence, the occurrence of air bubbles should be minimized in the coupling medium in order to avoid ultrasound reflection. The reflection coefficient, \( r \), for various interfaces may be estimated from the acoustic impedances of the two media forming the interface, \( Z_1 \) and \( Z_2 \), using the following equation [59, 60]:
\[ r = \left( \frac{Z_1 - Z_2}{Z_1 + Z_2} \right)^2 \]

In summary, ultrasound is a sound wave possessing frequencies above 20 KHz. Ultrasound is generated using a device referred to as a sonicator, and may be transmitted to the skin using water as a coupling medium. In the next chapter, details of the experimental methods utilized in this investigation of sonophoresis are discussed.
3. Experimental Methods

3.1. Skin Preparation for Permeation Experiments: Full thickness human cadaver skin (male/female abdominal or back skin) was obtained from local hospitals (Beth Israel Hospital, Boston or Bringham and Woman Hospital, Boston) or the National Disease Research Institute. The skin was kept frozen at -70°C for up to six months. Prior to experiments, the skin was defrosted by keeping it in water at room temperature for about 2 hours after which, the skin was heat-stripped. Heat stripping was performed by keeping full thickness skin in water at 60 °C for exactly two minutes, followed by removal of the epidermis by careful scraping using a spatula. Separated epidermis was floated on water and then placed on a bench-top paper and stored for up to 14 days in a desiccator at 4°C and 95% relative humidity until the experiments were performed. Ninety-five percent humidity was maintained in the desiccator by placing a supersaturated aqueous solution of potassium sulfate (Sigma Chemicals) in the bottom.

3.2. Transdermal Transport Measurements Using Radiolabelled Compounds: All the permeants studied, including estradiol, progesterone, testosterone, benzene, butanol, caffeine, corticosterone, sucrose, water, and aldosterone, were radiolabeled (either ³H or ¹⁴C, NEN Research Products). A section of heat-stripped skin was removed from the chamber just prior to the experiment, and mounted onto a diffusion cell with the stratum corneum side facing the donor compartment. Under therapeutic conditions, experiments were performed using a side-by-side diffusion cell custom-made with parts manufactured from Crown Glass Co. This cell was prepared using commercially available diffusion cell caps (FDC 400, Flat Flange) to which a small glass port was attached for sample withdrawal (Figure 3a). One end of the diffusion cell was sealed using an ultrasound absorber (generic teflon sheet of 1 mm thickness), and at the other end, an ultrasound transducer (1 or 3 MHz, 1 cm² area, sonopuls 434) was glued using epoxy. In the case of low-frequency sonophoresis, vertical FDC 400 diffusion cells (Crown Bioscientific) were used (Figure 3b). In these experiments, the skin was supported by a nylon mesh (Tetko Inc. part no. 3-150/151) to avoid any damage due to mechanical oscillations. Prior to mounting the mesh, a thin layer of vacuum grease (Dow Chemicals) was applied on the edge to avoid any leaks. It was made sure by visual observation, that the skin piece was resting on the mesh and not floating on the water layer supported by the mesh.
An Ag/AgCl electrode was introduced in each compartment prior to mounting the skin, and the electrical resistance of the skin was measured prior to each experiment according to methods described in section 3.4. If the specific resistance (= Resistance × Skin Area) was found to be less than 10 KΩ-cm² (indicative of skin damage) the skin piece was removed and replaced by another piece. Temperature in the diffusion cell was measured periodically using a thermocouple (Digi-thermo, VWR Scientific) on the skin surface as well as at other locations close to the skin in the diffusion cell. No spatial variation of temperature was detected. All experiments were performed at room temperature (25°C). The donor and receiver compartments were clamped together, and the receiver compartment was filled with Phosphate Buffer Saline (PBS, phosphate concentration = 0.01 M, NaCl concentration = 0.137M) (Sigma Chemical Co.). The donor compartment was typically filled with a 1μCurrie/ml solution of the permeant in PBS. The diffusion cell compartments were stirred using a magnetic stirrer at a speed of 100 rpm (800 rpm in the case of benzene transport). The concentration of the permeant in the receiver compartment was measured using a scintillation counter (model 2000 CA, Packard).

3.3. Transdermal Protein Transport Measurements: Transdermal transport of three proteins, insulin, γ-interferon, and erythropoietin, was measured in the presence and absence of low-frequency ultrasound (20 KHz, 100 msec pulses applied every second at intensities in the range of 0-225 mW/cm²). These experiments were performed using the experimental set-up shown in Figure 3b. In separate experiments, the donor compartment of the diffusion cell was
filled with a solution of insulin (100 U/ml, Humulin Regular, Eli Lilly (purchased from MIT pharmacy, Cambridge), γ-interferon (2500 U/ml, Genzyme Corp.), or erythropoietin (400 U/ml, Amgen Corp., purchased from Beth Israel Pharmacy store, Boston). Ultrasound (20 KHz, 100 msec pulses applied every second) was applied at intensities in the range of 0 mW/cm² - 225 mW/cm²) for 4 hours. The receiver compartment was filled with 2% BSA in PBS to minimize sticking of the proteins to glass. The concentration of the proteins in the receiver compartment was measured every hour either by Radio-Immuno Assay (RIA) or Enzyme-Linked Immuno-Sorbent Assay (ELISA). Insulin RIAs were performed at Linco Research Inc., St. Charles. The γ-interferon ELISAs were performed using a kit purchased from Endogen Inc., and the erythropoietin ELISA was performed at ARUP, Salt Lake City. For protein concentration measurements, about 500 µl samples were taken from the receiver compartment, stored at -20°C in a glass vial and shipped on dry ice for RIAs or ELISAs as described above.

3.4. Ultrasound Application

Therapeutic Ultrasound: Ultrasound was applied at frequencies of 1 or 3 MHz, and intensities of up to 2 W/cm² using an ultrasound generator (model Sonopuls 474, Henley International). The application of ultrasound was always continuous, rather than pulsed, unless mentioned otherwise. The transdermal flux in the presence of ultrasound was measured every hour. Both the donor and the receiver solutions were removed after four hours, gassed by bubbling air through the solutions using rubber tubing connected to the air tap, and placed back into the corresponding compartments. This procedure was followed to ensure a sufficiently high dissolved air concentration in the solutions. The transdermal flux in the presence of ultrasound decreased with time even though ultrasound was kept continuously ON. It was found that regassing the donor and receiver solutions every four hours maintains the transdermal flux at a steady value, which is referred to as the steady-state transdermal flux. The skin permeabilities in the presence of ultrasound, $P^{us}$, as well as those in the absence of ultrasound (passive permeability), $P^p$, were calculated from the steady-state transport rates in the presence and absence of ultrasound respectively [$P=j/\Delta C$, where $j$ is the steady-state transdermal flux, and $\Delta C$ is the concentration difference across the skin]. The pressure amplitude of ultrasound on the skin surface was measured using a hydrophone (model PZT 54, Specialty Engineering Associates) coupled to a hydrophone preamplifier (model A17DB, Specialty Engineering Associates), and finally connected to an oscilloscope (model 7623 A, Hewlett Packard). The hydrophone was calibrated by Sonic Technologies. The ultrasound intensity, $I$, (Spatial Average Temporal Peak) was calculated from the values of the acoustic pressure amplitude, $P$, measured using a hydrophone (Model 8103, Bruel and Kjaer) using the equation, $I = P^2 / 2\rho c$, where $\rho$ is the water density (1 gm/ml), and c is the velocity of ultrasound in water [1500 m/s].
**Low-Frequency Sonophoresis:** Ultrasound was applied using a sonicator (VCX 400, Sonics and Materials) operating at a frequency of 20 KHz. The ultrasound pressure amplitude was measured using a hydrophone (Model 8106, Brul and Kjaer), and the ultrasound intensity was calculated using the equation presented above. In order to avoid any thermal effects, a pulsed application of ultrasound (100 msec long pulses applied every second also referred to as 10% duty cycle) was chosen, although the choice of pulse rate was arbitrary. The temperature of the solutions in the diffusion cell was measured using a thermocouple (Digithermo, VWR Scientific). No significant increase in the diffusion cell temperature (< 2 °C) was observed upon ultrasound exposure. The ultrasound intensity was varied in the range of 0-225 mW/cm² in 4 steps (12.5 mW/cm², 62.5 mW/cm², 125 mW/cm², and 225 mW/cm²). The skin permeabilities in the presence of ultrasound, \( k_{\mu} \), as well as those in the absence of ultrasound (passive permeability), \( P^p \), were calculated from the steady-state transport rate in the presence and absence of ultrasound respectively [\( P = I/\Delta C \), where \( I \) is the steady-state transdermal flux, and \( \Delta C \) is the concentration difference across the skin].

**3.5. Skin Electrical Resistance Measurements:** Ag/AgCl electrodes (E242, Invivo Metrics) were introduced in the donor and receiver compartments in order to measure the skin electrical resistance. An AC electric field, typically at 50-100 mV and 10 Hz, was applied across the electrodes for a short time (typically, for 5 seconds) using a signal generator (model HP 4116 A, Hewlett Packard). The electric current through the skin was then measured using an ammeter (Texas Instruments), and the electrical resistance was calculated using Ohm's law. The saline resistance was measured separately using the same assembly, but without mounting the skin. Since the measured skin electrical resistance is the sum of the actual skin electrical resistance and the saline electrical resistance, the latter was subtracted from the measured skin electrical resistance to obtain the actual skin electrical resistance.

**3.6. Confocal Microscopy:** Confocal microscopy allows the optical cross-sectioning of a sample (for example, the SC) by deconvoluting fluorescence from various depths in the sample. This technique allows a better visualization of the inner layers of the SC. Skin samples to be observed under the confocal microscope were frozen immediately after the sonophoresis experiment. These samples were defrosted prior to the microscopic observations, mounted on a microscopic slide as flat as possible, and placed under a confocal microscope (model MRC-600, Bio-Rad). The samples were excited using a Krypton-Argon laser beam at a wavelength of 488 nm, and the emitted light signals were analyzed at a wavelength of 515 nm in order to generate a digitized micrograph.
3.7. In Vivo Experiments: *In vivo* experiments were performed to assess the efficacy of sonophoresis across living skin. Hairless rats (IFFA Credo and Charles River, about 16 weeks old, either sex) were used as an animal model for these studies, since it has been shown that the transport properties of hairless rat skin are comparable to those of human skin [63]. The hairless rats were anesthetized by an IP injection of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). Additional doses, each comprising 1/3 of the initial dose, were given every hour to sustain anesthesia. After about an hour into anesthesia, a flanged glass cylinder (Crown Glass Company, diameter 20 mm, height 2 cm) was glued on the rat's back using a minimal amount of superglue (Permabond International) or vacuum grease (Dow Chemicals) on the outer edge of the flange. The center of the cylinder was located about 3 cm from the rear end of the rat. This particular site was chosen to avoid application of ultrasound directly on a sharp bone close to the body surface, which otherwise might have caused damage to the blood capillaries near the edge of the bone. The cylinder was filled with a solution of radiolabelled salicylic acid (about 2 \( \mu \text{Ci/ml} \)) or insulin (100 U/ml). Ultrasound (20 KHz, 0-225 mW/cm\(^2\) (125 mW/cm\(^2\) in the case of salicylic acid) 100 msec pulses applied every second) was applied by immersing the transducer in the donor solution. The transducer was located about 1 cm away from the skin. The concentration of salicylic acid in the rat urine was measured using a scintillation counter (model 2000 CA, Packard). In the case of insulin transport experiments, the glucose level in the rat blood was measured using a commercially available device Accuchek Advantage (Boheringer Mannheim). Each blood glucose level was measured in triplicate to minimize errors due to the assay method. Blood was withdrawn from the tail vein by chopping off about 1 mm end portion of the tail. For insulin level measurements, about 400 \( \mu \text{l} \) of blood was collected in a centrifuge tube rinsed with a heparin solution to avoid coagulation. The blood was then centrifuged and plasma was sent on dry ice to Linco Research for assay.

3.8. Induction of Diabetes: Diabetes was induced by a single intravenous (tail vein) dose of streptozotocin (65 mg/Kg). Streptozotocin was prepared by dissolving 100 mg of powder (Boheringer Mannheim) in 2.5 ml of buffer prepared by dissolving 700 mg of sodium citrate and 550 mg of citric acid in 100 ml of water. The pH of the buffer was adjusted to 4.5 using NaOH or citric acid. Once made, the buffer was stored on ice. Streptozotocin was added to the buffer just prior to injection. Once made, the streptozotocin solution was used within 5 minutes. The blood glucose of the rats was checked every 12-15 hours. For the first 12 hours, glucose was added to the water (about 20-30 g/liter) supplied in the rat cages to avoid initial hypoglycemia that may be induced by streptozotocin. After 12 hours, normal water was given to the rats. The rats normally became diabetic in 24 hours (indicated by their blood glucose level above 400
mg/dL), however, 48-72 hours were allowed to ensure diabetic hyperglycemia (blood glucose level > 400 mg/dL).

3.9. **Histological Studies**: Histological studies of the hairless rat skin exposed to ultrasound were performed to assess the safety of low-frequency sonophoresis as a penetration enhancer. These studies were performed at Deborah Heart and Lung Institute, New Jersey. The hairless rat skin exposed to ultrasound was removed within 5 minutes after sacrificing the rat and stored in formalin. These samples were stained with hematoxylin and eosin (Deborah Heart and Lung Institute, New Jersey). The stained skin samples were later observed under a light microscope (40-fold magnification) to assess possible structural damage to the skin.
4. Therapeutic Sonophoresis
Mechanisms and Applications

4.1. Effect of Therapeutic Ultrasound on Transdermal Transport: Application of therapeutic ultrasound has been attempted since the middle of this century for local delivery of certain drugs. Nevertheless, only one drug, hydrocortisone, is currently delivered transdermally using therapeutic ultrasound in clinical applications. In view of the long precedence for the use of therapeutic ultrasound for sonophoresis, the initial studies of sonophoresis in this thesis were conducted using therapeutic ultrasound. The primary objectives of these experiments were:

i) To understand the mechanism responsible for sonophoresis, that is, to understand which of the four possible mechanisms, including cavitation (generation and oscillation of air bubbles), thermal effects (increase in temperature), acoustic streaming (generation of convective velocities), and mechanical effects (generation of mechanical stresses due to oscillating pressures), plays an important role in sonophoresis.

ii) To evaluate the effect of therapeutic ultrasound on the transdermal transport various model drugs.

iii) To develop mathematical models to predict therapeutic sonophoretic enhancements.

iv) To understand why therapeutic ultrasound enhances transdermal drug transport by less than 10-fold.

Application of ultrasound at 1MHz, 2 W/cm² (continuous) enhanced transdermal transport of a variety of permeants including estradiol, testosterone, corticosterone, salicylic acid, benzene, butanol, and progesterone. For example, Figure 4 shows a comparison of the cumulative amount of estradiol transported transdermally in the presence and absence of ultrasound (the amounts are reported as percent of the amount present in the donor compartment). The transport rate (flux) in both cases is proportional to the slopes of the corresponding curves. The control transdermal flux attains a steady value (indicated by a constant slope of the curve) within one hour of beginning the experiment. On the other hand, the transdermal flux in the presence of ultrasound exhibits a significant temporal variation. Initially, it is 13 (±1.5) fold higher than that of the control (enhancement ratio $E = P_{us}/P_p$, where $P_{us}$ is the sonophoretic permeability and $P_p$ is their passive permeability), and remains at this

26
value for about 4 hours. However, continued application of ultrasound after this initial four-hour period has a progressively smaller enhancement effect, and eventually (typically, after 6 hours) the transdermal estradiol flux in the presence of ultrasound becomes comparable to the control flux (the slopes of the two curves are equal).

It is possible to rationalize this reduction in sonophoretic enhancement if one hypothesizes that cavitation plays an important role in sonophoresis. Specifically, it is known that ultrasound causes degassing of the saline surrounding the skin due to cavitation [64]. Since dissolved air is necessary for cavitation, a decrease in the dissolved air concentration in the saline may cause a decrease in the cavitation activity. In order to check this hypothesis, additional sonophoresis experiments were performed in which the donor and the receiver solutions were removed every hour, air was bubbled through these solutions for five minutes, and the solutions were put back in their corresponding compartments. Results of these experiments are shown in Figure 5.

**Figure 4** - Variation of the transdermal estradiol flux with time during ultrasound exposure (1 MHz, 2 W/cm²): (●) In the presence of ultrasound, (○) In the absence of ultrasound (Passive controls). Typical error bars (SD) are shown on one data point.

**Figure 5** - Effect of regassing on the transdermal flux of estradiol in the presence of ultrasound (1 MHz, 2 W/cm²): (●) Donor solution regassed every hour, (○) No regassing, (■) Passive-controls. Typical error bars (SD) are shown on one data point.

The transdermal flux in the presence of ultrasound when the solutions are regassed every hour remains high well beyond the initial four-hour period in contrast to the leveling off when the solutions are not regassed. In fact, the observed flux increases after every regassing. It
was also found that while regassing every hour continuously increases the transdermal flux, when done every four hours, it maintains the transdermal flux at a steady value. Accordingly, the donor and the receiver solutions were regassed every four hours in all subsequent experiments. The observation that dissolved air content in the donor and receiver solutions plays an important role in the efficacy of ultrasound in enhancing transdermal transport suggests that cavitation may play a major role in sonophoresis. A detailed mechanistic study portraying the role of cavitation in sonophoresis is presented later.

Ultrasound under similar conditions (1MHz, 2 W/cm²) enhanced transdermal transport of a variety of other permeants, including testosterone, benzene, corticosterone, progesterone, caffeine, and butanol, to an extent that depends on the physico-chemical properties of the permeant. Table 2 provides a summary of the results of these experiments. These results show two characteristics: i) the enhancement varies from drug to drug, and ii) the highest enhancement is about 10-fold, observations which are consistent with the literature reports on sonophoresis (see Chapter 1). A mechanistic explanation for these characteristics of sonophoresis is provided next.

<table>
<thead>
<tr>
<th>Permeant</th>
<th>Molecular Weight (Da)</th>
<th>Passive Permeability ( P^p ) (cm/hr)</th>
<th>Partition Coefficient ( K_{octanol/water} )</th>
<th>Ultrasound Enhancement ( E )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>78</td>
<td>1.6 \times 10^{-1}</td>
<td>100</td>
<td>1 \pm 0.2</td>
</tr>
<tr>
<td>Butanol</td>
<td>162</td>
<td>2.2 \times 10^{-3}</td>
<td>7.5</td>
<td>1.5 \pm 0.5</td>
</tr>
<tr>
<td>Caffeine</td>
<td>194</td>
<td>1.0 \times 10^{-4}</td>
<td>1</td>
<td>1.2 \pm 0.4</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>346</td>
<td>3.0 \times 10^{-4}</td>
<td>8</td>
<td>4 \pm 0.6</td>
</tr>
<tr>
<td>Estradiol</td>
<td>272</td>
<td>3.2 \times 10^{-3}</td>
<td>7000</td>
<td>13 \pm 1.5</td>
</tr>
<tr>
<td>Progesterone</td>
<td>274</td>
<td>1.3 \times 10^{-2}</td>
<td>6000</td>
<td>1 \pm 0.5</td>
</tr>
<tr>
<td>Testosterone</td>
<td>288</td>
<td>2.2 \times 10^{-3}</td>
<td>2070</td>
<td>5 \pm 1.1</td>
</tr>
</tbody>
</table>

Table 2-Effect of therapeutic ultrasound on transdermal drug transport.

4.2. Mechanism of Sonophoresis: Many phenomena, including cavitation, thermal effects, generation of convective velocities, and mechanical effects, have been considered to play a role in the ultrasound-mediated enhancement of transdermal transport. A detailed description of the possible role played by each of the mechanisms, and the experiments performed to elucidate their relative importance in sonophoresis is described below. For the mechanistic investigations of therapeutic sonophoresis, estradiol was chosen as a model permeant since the highest
sonophoretic enhancement was observed in this case (Table 2). Detailed experimental procedures are discussed in Chapter 3.

4.2.1. Thermal Effects: The increase in the skin temperature resulting from the absorbance of ultrasound energy may increase the skin permeability coefficient due to an increase in the permeant diffusion coefficient. In a different set of experiments, it was found that a temperature increase of 10 °C causes about a 2-fold increase in the skin estradiol permeability. This observation is also in agreement with the literature reports [65] suggesting about a 2-fold increase in the skin permeability coefficient per 10 °C rise in the skin temperature. Since the typical skin temperature increase in the sonophoresis experiments is about 70°C, it can be concluded that thermal effects cannot explain the observed 13-fold enhancement of estradiol transdermal transport upon ultrasound exposure.

4.2.2. Convective Transport: Fluid velocities are generated in a porous medium exposed to ultrasound for a variety of reasons, including the interference of the incident and reflected ultrasound waves in the diffusion cell, and oscillations of the cavitation bubbles. Fluid velocities generated in this way may affect transdermal transport by inducing convective transport of the permeant across the skin, especially through hair follicles and sweat ducts. In order to assess the validity of this hypothesis, electrical resistance measurements of the skin exposed to ultrasound were performed. It is known that the mobility of ions in the skin is sufficiently high such that the electrical resistance measured using moderate electric fields constitutes an almost instantaneous indicator of the skin transport properties [66]. In addition, the time required to establish convective currents in the donor compartment or in the follicles (hair follicles and sweat ducts) of the skin (both filled with buffer) upon ultrasound exposure is expected to be fairly short (of the order of seconds) due to the low viscosity of water [67]. Accordingly, if exposure to ultrasound changes the skin electrical resistance, and if the occurrence of convective transport is reflected in the change in skin electrical resistance, one may expect to observe a rapid change in the skin electrical resistance. Figure 6 shows a typical measured variation of skin electrical resistance, \( R \), (normalized by the initial resistance, \( R_0 \)) with time upon ultrasound application (1 MHz, 2 W/cm²). Results from a single, typical experiment are shown in order to clearly depict the shape of the curve. The electrical resistance of the skin decreases upon ultrasound exposure due to increased ionic mobility.
Figure 6 - Variation of the electrical resistance, $R$, of the skin (normalized by the initial resistance, $R_0$) with time upon ultrasound exposure.

Figure 7 - Relationship between the sonophoretic skin permeability to molecules listed in Table 2 and their lipophilicities represented in terms of their octanol-water partition coefficients, $K_{ow}$.

As shown in Figure 6, the electrical resistance decreases by about 30% over 45 minutes. The decrease is nearly exponential, and far from rapid, suggesting that convective transport may not play an important role in the observed enhancement. In addition, it may be hypothesized that if any convective transport occurs through hair follicles or sweat ducts one may expect that the sonophoretic skin permeability should be independent of drug lipophilicity since the follicles are likely to be filled with saline surrounding the skin. However, it was observed that the sonophoretic permeability is proportional to the lipophilicity (measured by octanol-water partition coefficient) of drugs (Figure 7). These results suggest that transdermal transport of drugs during therapeutic sonophoresis occurs through lipid bilayers of the skin and not hair follicles.

4.4.3. Mechanical Stresses: Ultrasound is a longitudinal pressure wave inducing sinusoidal pressure variations in the skin. Nevertheless, there is no significant net pressure gradient across the SC at any time because the thickness of the SC (15 μm) is very small compared to a typical ultrasound wavelength (1500 μm at 1 MHz). In view of this, little attention has been paid to the investigation of mechanical effects as a possible mechanism of sonophoresis. The sinusoidal pressure variations induce sinusoidal density variations in the medium. At low ultrasound frequencies, these density variations can grow into a gas or vapor bubble, thus giving rise to
cavitation. However, at higher frequencies, where the density variations occur so rapidly that a small gaseous nucleus cannot grow, cavitation effects cease, although other effects due to density variations may continue to occur. These effects include primarily the generation of cyclic stresses due to density changes ultimately leading to fatigue of the medium. Lipid bilayers, being self-assembled structures, can be easily disordered by these stresses, thereby increasing the bilayer permeability.

The hypothesis that mechanical stresses play an important role in the therapeutic ultrasound range is inconsistent with the previously mentioned observation that the effect of 1 MHz ultrasound on transdermal transport decreases with time due to degassing of the solutions in the diffusion cell (Figure 4), since the mechanical effects may continue to occur in the skin in spite of the fact that the surrounding solution is degassed. In addition, it is known from investigations of the effect of cyclic stresses on polymers and metals, that the mechanical effects are proportional to the stress frequency [68]. Since the frequency of cyclic stresses (if any) in a medium exposed to ultrasound may follow the ultrasound frequency, one would expect that if mechanical effects play a major role in sonophoresis, ultrasound at higher frequencies should be at least equally efficacious in enhancing transdermal transport. However, it was found that while 1 MHz ultrasound enhanced estradiol transdermal transport by a factor of 13, 3 MHz ultrasound at the same intensity (2 W/cm²) enhanced transport only by 50% (these results are discussed later in detail). These observations suggest that mechanical effects do not play an important role in sonophoresis using ultrasound in the therapeutic frequency range.

4.2.4. Cavitation: Cavitation involves the generation and oscillation of gaseous bubbles in a medium, and may be induced by the exposure to ultrasound. Cavitation occurs due to the nucleation of small gaseous cavities during the negative pressure cycles of ultrasound, followed by the growth of these bubbles throughout subsequent pressure cycles [55]. It is noteworthy that whenever small gaseous nuclei already exist in a medium, cavitation takes place preferentially at those nuclei [55]. The minimum ultrasound intensity required for the onset of cavitation is referred to as the cavitation threshold, and increases rapidly with ultrasound frequency. No significant cavitation effects have been observed in fluids at high ultrasound frequencies (frequency > 2.5 MHz), even at intensities which are much higher than those corresponding to therapeutic ultrasound [69]. As a result, 2.5 MHz is considered to be a reasonable estimate of the upper frequency limit for the occurrence of cavitation in fluids at therapeutic ultrasound intensities. The cavitation threshold also depends significantly on the ultrasound pulse length. For example, the cavitation threshold in an aqueous solution at 1 MHz changes from approximately 0.3 W/cm² [55] to 33 W/cm² [70] as the mode of ultrasound
application changes from continuous to pulsed, with a pulse length of 1 msec applied every 10 msec.

Experiments were performed to determine if the dependence of sonophoresis on ultrasound frequency and pulse length follows a trend similar to that of the cavitation activity in fluid media. The results of these experiments are presented in Figure 8, and may be used to test the hypothesis that cavitation is a possible mechanism of sonophoresis. While 1 MHz ultrasound (2 W/cm², continuous) resulted in a 13-fold enhancement of the transdermal estradiol flux, 3 MHz ultrasound (2 W/cm², continuous) resulted in only a 50 % enhancement (compare cases 1 and 2 in Figure 8).

![Figure 8](image)

**Figure 8**- Effect of ultrasound on the transdermal estradiol transport under a variety of conditions: case 1: 1 MHz from normal buffer, case 2: 3 MHz from normal buffer, case 3: 2 msec pulsed application from normal buffer, case 4: 1 MHz compressed skin, case 5: 1 MHz degassed skin, and case 6: 1 MHz form viscous solvent. Cases 2, 3, 4, and 5 suggest that cavitation in the skin plays a major role in sonophoresis, while case 6 suggests that cavitation outside the skin does not play any role in sonophoresis.

Furthermore, application of 2 msec ultrasound pulses (one pulse every 10 msec) at 2 W/cm² and 1 MHz did not cause any enhancement of estradiol transdermal transport (case 3 in Figure 8). These results are consistent with the above mentioned dependence of cavitation in fluids on ultrasound parameters, and indicate that cavitation may play an important role in the observed ultrasound-mediated transdermal transport enhancement.

Cavitation may occur either inside the skin (in particular, inside the stratum corneum), outside the skin, or in both domains. Accordingly, each possibility was examined in detail as described below.
Cavitation Inside the Skin as a Possible Sonophoresis Mechanism: Cavitation occurs in a variety of mammalian tissues, including muscle, abdominal tissues, brain, cardio-vascular tissues, and liver upon exposure to ultrasound at a variety of conditions which include the therapeutic range discussed above [71]. As explained earlier, the occurrence of cavitation in biological tissues is attributed to the existence of a large number of gas nuclei [71] These nuclei are gas pockets trapped in either intracellular or intercellular structures. In order to determine whether cavitation occurs inside the skin, two sets of experiments were performed.

In the first set, attempts were made to utilize the known effect of static pressure on cavitation. It has been shown that cavitation in fluids and porous media [72] can be suppressed at high pressures. This effect is believed to occur due to the dissolution or collapse of the gaseous nuclei under the influence of pressure. Based on this known effect, experiments were designed where pieces of heat-stripped human cadaver skin were compressed prior to the sonophoresis experiment. This was achieved by placing the skin pieces between two smooth glass plates soaked in water, and then compressing the entire assembly using a compression press at a pressure of 30 atm for two hours. The skin pieces were then removed, and permeability experiments were performed according to the protocols described in chapter 3. Control experiments were also performed on compressed skin. It was found that compression reduces the control flux by 50% compared to normal controls. When the compressed skin piece was exposed to ultrasound (1 MHz, 2 W/cm²), the transdermal transport was enhanced only by 75% in contrast to the 13-fold increase found in the case of uncompressed skin (compare cases 1 and 4 in Figure 8). (Note that the control flux used to calculate the enhancement ratio in case 4 was also measured using compressed skin).

In the second set of experiments, heat-stripped human cadaver skin was degassed prior to the permeability experiments. It was anticipated that when a skin piece soaked in buffer is subjected to high vacuum, the resulting low pressures should reduce the dissolved gas concentration in the buffer thereby forcing small gaseous nuclei in the skin to dissolve. Degassing of the skin was achieved by placing the skin in buffer and maintaining a vacuum of 0.05 mm Hg over it for two hours. After degassing was done, the skin was removed and permeability experiments were performed according to the protocols described in Chapter 3. Control fluxes were also measured using degassed skin, and were found to be about 40% higher than those corresponding to the normal control fluxes. This probably occurs due to structural changes in the SC lipid bilayers under high vacuum. When the degassed skin was exposed to ultrasound, once again, the effect of ultrasound on the permeability of degassed skin was minimal (50%), thus supporting the hypothesis that cavitation inside the skin plays a major role in enhancing transdermal transport upon ultrasound exposure (case 5 in Figure 8. Note that the control flux used to calculate the enhancement ratio was also measured using degassed skin).
The occurrence of cavitation inside the skin was investigated further, with emphasis on its location within the skin (stratum corneum, in particular) as well as its effect on transdermal transport.

**Cavitation Outside the Skin as a Possible Sonophoresis Mechanism.** Experiments were performed to assess the importance of cavitation outside the skin. Cavitation in the saline surrounding the skin does occur after ultrasound exposure. Indeed, the resulting cavitation bubbles can be seen by the naked eye. These cavitation bubbles can potentially play a role in the observed enhancement of the transdermal transport in a number of ways. First, these bubbles may cause skin erosion following their violent collapse on the skin surface due to the generation of shock waves [73], thereby enhancing transdermal transport. Second, the oscillations and collapse of cavitation bubbles may also cause generation of velocity jets at the skin-donor solution interface. This phenomenon is referred to as microstreaming [74], and may induce convective transport across the skin, thereby enhancing the overall transdermal transport.

In order to assess the importance of cavitation outside the skin, the well-known fact that cavitation activity in a fluid medium decreases as the viscosity of the medium increases was utilized. This probably occurs due to a decrease in the ability of gases to diffuse from the bulk fluid to the nucleation sites [71]. The effect of ultrasound on the transdermal flux of a permeant was studied from two different donor solutions. In the first case, the donor solution consisted of estradiol in PBS (viscosity of about 0.9 cp). In the second case, the donor solution consisted of estradiol in a water soluble viscous gel (Ployfrozez, Polyscience, viscosity of more than 100 cp). The receiver compartment was filled with PBS in both cases. Ultrasound (1 MHz, 2 W/cm²) was applied as described in Chapter 3. Control experiments indicated that the passive transdermal transport rate is not affected by the fact that the donor solution viscosity is high. This is plausible, since the rate limiting step in permeant transport from the donor compartment to the receiver compartment is always the diffusion through the skin and not the diffusion in the donor compartment. Upon ultrasound exposure, no cavitation bubbles were observed in the gel, which are otherwise clearly visible if the donor compartment contains an estradiol solution in PBS. Nevertheless, the ultrasound-induced transdermal transport enhancement in this case was found to be comparable to that when the donor compartment contains estradiol solution in PBS (compare cases 1 and 6 in Figure 8). This finding suggests that cavitation outside the skin may not play an important role in sonophoresis using the ultrasound conditions examined.

In the next section, the results of additional experiments aimed at elucidating the location of cavitation inside the stratum corneum are discussed.
4.3. Location of Cavitation Inside the SC: Cavitation inside the SC can potentially take place in the keratinocytes, or in the lipid regions, or in both places. To assess the location of cavitation inside the SC as well as to obtain further evidence for the occurrence of cavitation inside the SC, the following additional experiments were performed.

It has long been known that cavitation in an aqueous medium leads to the formation of hydroxyl radicals after adiabatic collapse of the cavitation bubbles [55]. The hydroxyl radicals being very unstable react with water to from hydrogen peroxide, a strong oxidizing agent. In a different set of experiments, it was found that hydrogen peroxide oxidizes fluorescein, a highly fluorescent dye, into a non-fluorescing compound. On the basis of this observation and the known fact that ultrasound exposure in water leads to the formation of hydrogen peroxide, one may expect that an exposure to ultrasound should result in a significant bleaching of fluorescein. Indeed, as can be seen in Figure 9, a significant amount of fluorescein is bleached upon continuous exposure of its aqueous solution (1μg/ml) to ultrasound (1 MHz, 2 W/cm²).

The initial rate of bleaching is high, although it decreases with time probably due to reduced cavitation activity. It was also found that 3 MHz ultrasound at the same intensity does not induce any bleaching. This suggested that the bleaching in the presence of ultrasound was related to cavitational effects. Based on this observation, it was anticipated that if significant cavitation activity occurs in the SC upon ultrasound exposure, one should see a similar bleaching of the fluorescence in the SC pre-loaded with fluorescein.

![Graph](image)

**Figure 9**-Variation of the amount of fluorescein bleached as a function of the ultrasound exposure time. This bleaching occurs due to oxidation of fluorescein by cavitation-generated peroxide radicals. Results from a single experiment are shown to depict the shape of the curve clearly. The typical error in the data is 15%.
Pieces of heat-stripped human cadaver skin were soaked in a 1μg/ml solution of fluorescein. The solution was kept at 4 °C to ensure minimal skin degradation during storage. After 5 days of soaking, these samples were observed using a fluorescent confocal microscope. Methods used for confocal microscopy are discussed in Chapter 3. Figure 10a shows a typical confocal micrograph revealing the well-known honey-comb like structure of the SC. The hexagonal structures are the keratinocytes, and the bright contiguous borders around the keratinocytes are the intercellular lipid bilayers. Note the presence of fluorescein (proportional to the brightness in the micrograph) in both the keratinocytes and in the intercellular lipid bilayers.

Ultrasound (1 MHz, 2 W/cm²) was applied to similar skin pieces (soaked in 1 μg/ml fluorescein) by direct contact between the ultrasound transducer and the fluorescein solution in which the skin pieces were soaking. Ultrasound was turned OFF after 30 minutes. The skin pieces were washed, and immediately frozen in liquid nitrogen in order to minimize fluorescein transport after the exposure. Later, these skin pieces were observed using the confocal microscope. The fluorescence in the keratinocytes was significantly bleached, while the fluorescence in the intercellular lipids was not seriously affected (see Figure 10b).

![Figure 10a](image1)
![Figure 10b](image2)

**Figure 10a**-Confocal micrograph of the skin loaded with fluorescein. Note the significant amount of fluorescein in the keratinocytes (proportional to the brightness in the micrograph).

**Figure 10b**- Confocal micrograph of the skin exposed to ultrasound for 30 minutes. Note that a significant amount of fluorescein in the keratinocytes is bleached, thus suggesting occurrence of cavitation activity in the keratinocytes.

Before concluding that the observed bleaching of fluorescein in the keratinocytes was due to cavitational effects, the possibility that ultrasound caused the transport of fluorescein out of the SC, thereby causing a reduction of fluorescence brightness in the keratinocytes was considered. However, this event is not likely to happen during a 30-minute exposure to
ultrasound since the diffusion coefficient of fluorescein in the lipid bilayers is expected to be low (the passive diffusion coefficient of fluorescein is about $5 \times 10^{-10}$ cm$^2$/s, as estimated from the measured passive permeability coefficient and using methods for calculating the diffusion coefficient described in [75]). Furthermore, the skin was always surrounded by the fluorescein solution with which it was equilibrated for five days. Hence, there was no significant concentration gradient across the skin at any time. Accordingly, the loss of fluorescence upon ultrasound exposure can be attributed to the bleaching effect.

In order to confirm that the bleaching was related to cavitational effects, similar experiments were performed using 3 MHz ultrasound (2 W/cm$^2$), and found that 3 MHz ultrasound did not bleach fluorescein in the skin. These findings strongly support the hypothesis that ultrasound induces cavitation in the keratinocytes. However, it should be noted that these findings do not rule out the possibility of the occurrence of cavitation in the intercellular lipids.

4.4. Mechanism of Therapeutic Sonophoresis: Based on the discussions presented in the previous sections, it appears that ultrasound exposure in the therapeutic range causes cavitation in the keratinocytes of the stratum corneum. Oscillations of the ultrasound-induced cavitation bubbles, possibly near the keratinocyte-lipid bilayer interfaces, may, in turn, cause oscillations in the lipid bilayers, thereby causing structural disorder of the SC lipids. Shock waves generated by the collapse of cavitation bubbles at the keratinocyte-bilayer interfaces may also contribute to the structure-disordering effect.

Since the diffusion of permeants through a disordered bilayer phase can be significantly higher than that through a normal bilayer, transdermal transport in the presence of ultrasound is expected to be higher than passive transport (Figure 11).

![Figure 11](image)

**Figure 11:** Schematic representation of cavitation occurring in the keratinocytes. Cavitation occurs preferentially at the interface between the keratinocytes and the lipid bilayers.
In the next section, a quantitative description of sonophoresis based on the above proposed mechanism of sonophoresis is provided. The objectives of the quantitative analysis of sonophoresis are three fold: i) to predict sonophoretic enhancement for various drugs, ii) to understand the variation of the efficacy of sonophoresis from drug to drug, and iii) to understand why a typical enhancement induced by therapeutic ultrasound is 10-fold or less.

4.5. Quantitative Model of Therapeutic Sonophoresis: The passive skin permeability coefficient, $P^p$, is defined as the ratio of the molecular flux and the concentration difference across a uniform-thickness sample of SC. Under steady-state conditions, $P^p$ may be expressed as shown below [75]:

$$P^p = \frac{D^*}{h} \left[ K \right]$$  \[1\]

where $K$ is the SC-donor solution partition coefficient of the permeant, $D^*$ is the effective permeant diffusion coefficient in the SC (cm²/s), and $h$ is the SC thickness ($15 \times 10^{-4}$ cm). Since the passive transdermal transport of permeants occurs mainly through intercellular lipids, $K$ can be related to the partition coefficient of the permeant in the bilayer, $K_m$, and $D^*$ can be related to the permeant diffusion coefficient in the bilayers, $D_o$, through geometric parameters. These parameters include the fractional skin area occupied by the lipids (estimated value of 0.19 % [75]), and the ratio of the average length of the tortuous intercellular pathway to the SC thickness (estimated value of 24 [75]). For a detailed discussion of the estimation of these geometric parameters, see Ref. [75]. Substituting characteristic values for the SC geometric parameters in Eq. [1], and representing $P^p$ in units of cm/h (note that the diffusion coefficient units are cm²/s) one finds that:

$$P^p = 0.0019K_m \left[ \frac{D_o \times 3600}{(24) \times (15 \times 10^{-4})} \right] = 194K_mD_o \text{(cm/h)}$$  \[2\]

Similarly, the effective electrical conductivity of the skin, $\sigma$, can be described theoretically on the basis of the transport of ions such as sodium and chloride (present in the saline solution around the skin) through the shunt pathways as well as through the intercellular lipids. Specifically, $\sigma$ is given by Eq. [3] below. (for a detailed discussion of the derivation of this equation, see Ref. [75]).
\[ \bar{\sigma} = 1.2(1.2 \times 10^{-5} + 2.34 \times 10^{-4}\Phi) \quad (\Omega \text{ m})^{-1} \]

In Eq. [3], the first term in the brackets represents the contribution of the shunt pathways, and the second term represents the ionic motion along the intercellular lipid bilayers. \( \Phi \) is a function that expresses the hindrance of ionic motion as the ions pass through the confined regions between the head groups of the lipid bilayers of the stratum corneum. \( \Phi \) can be estimated using the analytical results of Faxen et al. [76] and Glandt [77], which respectively describe the transport and partitioning of spheres through a narrow rectilinear channel bounded by parallel walls. For unaltered configurations of the stratum corneum lipids, the lipid head groups from adjacent bilayers have been observed to be in extremely close proximity (the average bilayer separation is about 7 Å [78]). Under these conditions, assuming the hydrated radius of an ion such as sodium to be 3 Å, \( \Phi \) has been estimated by Edwards et al. [75] to be approximately 0.08. For a detailed discussion of the estimation of this parameter, see Ref. [75].

Exposure to ultrasound gives rise to cavitation in the keratinocytes of the SC. The existence of these cavitation bubbles and their oscillations in the presence of the propagating acoustic waves, may cause disordering of the intercellular lipids in the immediate vicinity of the keratinocytes. The intercellular lipid domains of the stratum corneum are assumed to be subdivided into regions of ordered lipid bilayers having fractional thickness, \( 1-f \), and disordered lipid regions having fractional thickness, \( f \). Because of the cavitation activity, a certain amount of water is expected to penetrate in the disordered lipid regions. This may lead to a reduction of the partition coefficient of lipophilic molecules in the disordered bilayers. The ionic transport through the disordered lipid bilayer regions may occur through these aqueous regions and hence is assumed to be essentially unhindered (\( \Phi = 1 \)). The diffusion coefficient of permeants through this region is assumed to be equal to that in free hydrocarbon, \( D^w \). The permeant partition coefficient in the bilayer, \( K_m \), is expected to increase upon disordering by less than one order of magnitude. However, water penetration in the disordered lipid regions may lead to a reduction of the partition coefficient of lipophilic molecules in the disordered bilayers. Because of these two opposing effects, the partition coefficient of the permeant in the bilayers may not change significantly upon bilayer disordering. In addition, as will be shown later, the permeant diffusion coefficients in an intact bilayer, \( D^w \), and that in a disordered bilayer, \( D_o \), differ typically by a few orders of magnitude. Hence, the effect of bilayer disordering on the drug diffusion coefficient is much larger than the possible effect on the partition coefficient. In view of this, in this analysis, the change in the permeant partition coefficient upon bilayer disordering are accounted for.

Based on the above description, Eqs. [2] and [3] can be expressed as follows
\[ P_{u} = 194K_{m}[fD^\infty + (1-f)D_o] \text{ (cm/h)} \]  \hspace{1cm} [4]

and

\[ \bar{\sigma}_{u} = 1.2[1.2 \times 10^{-5} + 2.34 \times 10^{-4}(f + (1-f)\Phi)] \text{ (\Omega m)^{-1}} \]  \hspace{1cm} [5]

Equation [4] describes the skin permeability coefficient to permeants in the presence of ultrasound, and Eq. [5] represents the corresponding skin electrical conductance. Dividing Eq. [4] by Eq. [2], and Eq. [3] by Eq. [5], yields the following expressions for the permeability enhancement ratio in the presence of ultrasound, \( E \), and for the electrical resistance reduction ratio, \( R/R_0 \):

\[ E \equiv \frac{P_{u}}{P_p} = 1 + f \left[ \frac{D^\infty}{D_o} - 1 \right] \]  \hspace{1cm} [6]

and

\[ \frac{R}{R_o} \equiv \frac{\bar{\sigma}}{\bar{\sigma}_{u}} = \frac{2.81}{1 + 19.5[f + 0.08(1-f)]} \]  \hspace{1cm} [7]

Equations [6] and [7] are simple analytical expressions that may be used to predict the transdermal permeability enhancement and the transdermal resistance drop in the presence of therapeutic ultrasound as a function of the transport properties of the permeant under consideration. These predictions require knowledge of the transport coefficients, \( D_o \) and \( D^\infty \), as well as of the fractional thickness of the lipid region disordered due to ultrasound application, \( f \). The lateral passive diffusion coefficient, \( D_o \), of the permeant through the stratum corneum intercellular lipids may be deduced from Eq. [2] by measurement of the passive transdermal permeability coefficient and a knowledge of the permeant partition coefficient in lipids, \( K_{m} \), (which can be approximately estimated using the correlation \( K_{m} = K_{o}/\omega^{0.75} \) [79]). The bulk diffusion coefficient, \( D^\infty \), can be measured in a representative alkane (for example, octane) or estimated on the basis of an established empirical formula [80]. Finally, \( f \) can be estimated from Eq. [7] via measurements of the transdermal resistance drop induced by ultrasound as discussed below.

Equation [7] predicts that if the entire lipid bilayer region is disordered, that is, if \( f = 1 \), the electrical resistance of the skin should drop by a factor of about 8. Accordingly, in order to ascertain whether Eq. [7] describes the phenomenon of bilayer structural disordering correctly, the following experiments were performed.

The electrical resistance of the skin was measured during a long (7 hour) exposure to ultrasound. As described earlier, a continuous application of ultrasound causes degassing of the solutions, thus making further exposure to ultrasound ineffective. In order to overcome this
problem, the donor and receiver solutions were regassed every hour. Figure 12 shows the variation of $R/R_0$ with time. The skin electrical resistance decreases upon ultrasound exposure and tends to plateau after about 45 minutes (this plateau is not seen clearly in Figure 12. For a clearer view of this plateau, see Figure 6). However, after regassing, the electrical resistance further decreases significantly.

This observation is consistent with the earlier observations that the regassing procedure maintains the effect of ultrasound on estradiol permeability coefficient at a high value. The observation that the regassing procedure maintains the effect of ultrasound on the skin electrical resistance at a high value provides additional support for the hypothesis that cavitation occurs inside the SC. Another feature of the variation of the electrical resistance shown in Figure 12 is that the effect of regassing decreases with time and reaches a plateau corresponding to a drop of resistance by a factor of 5 ± 1.5. This observation is in close agreement with the prediction of Eq. [7] that the maximum decrease in electrical resistance should approach a factor of 8. This finding supports the use of Eq. [7] to represent the structural changes that take place in the SC during ultrasound exposure.

The fraction of lipid bilayer disordered due to ultrasound exposure depends significantly on the number of regassings. In the permeation experiments, the donor and the receiver solutions were regassed every four hours. Hence, in most of our permeation experiments, total disordering of the lipid bilayers probably did not occur. In order to estimate the fraction of the lipid bilayers disordered in our permeation experiments, additional electrical resistance measurements were performed under exactly the same conditions as those corresponding to the permeation experiments discussed earlier (ultrasound frequency of 1 MHz, intensity of 2 W/cm², donor and receiver solutions regassed every four hours). It was found that, typically, the electrical resistance drops to about 60± (10)% of its initial value over the duration of an experiment. It is important to note that the skin electrical resistance may also drop because of the increase in skin temperature. Literature reports suggest that the skin electrical resistance decreases by 1-2% per °C increase in temperature [81]. In the sonophoresis experiments, it was found that the skin electrical resistance drops by about 14% when the skin temperature increases by 7°C (a typical increase in the skin temperature during our sonophoresis experiments), suggesting that 14% of the skin electrical resistance drop upon ultrasound exposure may be attributed to thermal effects. However, since the contribution of thermal effects to ultrasonic permeation enhancement has been neglected in the derivation of Eq. [6] for sonophoretic permeability enhancement, the entire electrical resistance drop is also attributed to cavitational effects. Substitution of $\frac{\sigma}{\sigma^u} = 0.6 (±0.1)$ in Eq. [7] results in a value of $f=0.13±0.05$ This value suggests that only about 13% of the lipid bilayer region surrounding the keratinocytes is disordered by the cavitation bubbles generated during the therapeutic.
ultrasound application. This small fraction of disordered bilayer also suggests that cavitation probably occurs in the keratinocytes of the SC. If any significant cavitation had occurred in the intercellular lipids, one would expect that a much larger fraction of the lipids should be disordered.

![Graph showing R/R0 over time](image)

**Figure 12-** Effect of long-time ultrasound exposure on the skin electrical resistance, R/R<sub>0</sub>. The electrical resistance drops after every regassing (done every hour) and finally levels off at a value of about 0.2 corresponding to a lowering of the skin electrical resistance by a factor of 5. A typical error bar is shown on one data point.

Figure 13 shows the theoretically predicted (solid lines indicating the upper and lower estimates of the enhancement corresponding to f=0.18 and f=0.08, respectively), as well as the experimentally measured values (circles) of the ultrasonic transdermal transport enhancement, E, as a function of the ratio, D<sup>-</sup>/D<sub>0</sub>. The theoretical predictions in Figure 13 are based on the simple formula that can be obtained by substituting f=0.18 or f=0.08 in Eq. [6], that is,

\[ E = (1 - f) + f \left[ \frac{D^*}{D_0} \right] \]  

[8]

As described earlier, values of D<sub>0</sub> can be obtained by substituting in Eq. [2] the experimentally measured passive skin permeability values, and the SC lipid partition coefficient values estimated using the correlation K<sub>m</sub> = K<sub>0</sub>/ω<sup>0.75</sup> [79]. The bulk diffusion coefficient of a permeant, D<sup>-</sup>, is assumed to be equal to the diffusion coefficient in free hydrocarbon (in this case, octane), and is estimated using the empirical formula (Wilke-Chang equation) described in Ref. [80].

The agreement between theory and experiment shown in Figure 13 is especially significant in view of the fact that no fitted parameters are used in the theoretical predictions.
As predicted by Eq. [6], the molecules diffusing passively at a fast rate (for example, benzene) are unaffected by therapeutic ultrasonic exposure, whereas those diffusing slowly (for example, estradiol) are significantly affected. These findings have important consequences regarding the efficacy of therapeutic ultrasound-mediated transdermal drug delivery. They indicate that the slower the diffusion of a permeant through the lipid bilayers of the SC (as reflected by its low $D_0$ value), the more effective is ultrasound in enhancing its transport. It is noteworthy, that since the skin permeability coefficient to a compound is governed by its partition coefficient as well as by its diffusion coefficient, the slowest permeating molecules are not necessarily the slowest diffusing ones. For example, the permeability coefficient of caffeine is one order of magnitude smaller than that of estradiol. Nevertheless, caffeine diffuses through the lipid bilayers of the SC at a rate which is two-orders of magnitude higher than that of estradiol. Accordingly, whereas caffeine permeates more slowly through the SC than estradiol, ultrasound is more effective at enhancing the permeation of estradiol.

This model also provides an explanation for the typical 10-fold or smaller enhancements induced by therapeutic ultrasound. A typical slow permeating drug has a passive diffusivity of $1 \times 10^{-8}$ cm$^2$/s (obtained using Eq. [2] and permeability data provided in Table 2).

![Graph](image.png)

**Figure 13**- Comparison of the theoretically predicted ultrasound-mediated transdermal transport enhancement values, $E$, (solid lines indicate the upper and the lower limits on the theoretical predictions) and the experimentally measured values (circles) plotted against the ratio, $D^\infty/D_0$.

The typical diffusivity of these drugs in a disordered medium is about $1 \times 10^{-6}$ cm$^2$/s, suggesting that the diffusivity of these molecules increases by 100-fold upon bilayer disordering. However, since only about 13% of the SC lipid bilayers are disordered by ultrasound application, the enhancement is restricted to about 10-fold. Therefore, a typical
sonophoretic enhancement induced by therapeutic ultrasound is of the order of 10-fold, although the precise enhancement varies from drug to drug.

4.6. Quantitative Analysis of Phonophoresis and Sonophoresis Literature: [82] It is possible that the observed variation of the efficacy of ultrasound-mediated transdermal drug delivery reported by various investigators is of a similar kind as that discussed above. In order to predict sonophoretic enhancements for drugs used in the past by various investigators, Eq. [8] was modified, by substituting Eq.[2] with $K_m = K_{0/w}^{0.75}$ and $f=0.13$, to arrive at the following equation to predict $E$:

$$E = 0.87 + \frac{K_{0/w}^{0.75}}{4 \times 10^4 P^p}$$  \[9\]

Equation [9] can be further simplified by subtracting 0.87 from both sides and referring to $E-0.87$ as the excess sonophoretic enhancement, $E'$. Specifically,

$$E' = \frac{K_{0/w}^{0.75}}{4 \times 10^4 P^p}$$  \[10\]

Equation [10] offers a simple expression to predict sonophoretic enhancements for any given drug based on the two experimentally measurable properties, $P^p$ and $K_{0/w}$.

Table 3 summarizes reports of therapeutic sonophoresis performed by various investigators [82]. Figure 14 shows a comparison of the $E'$ values predicted using Eq. [10] with the experimental values reported by various investigators.

![Diagram showing predicted E' value for various drugs](image)

**Figure 14**- Comparison of the model predictions of $E'$ values with the experimentally observed sonophoretic enhancements.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Experimental System</th>
<th>Ultrasound Conditions</th>
<th>Molecular Weight (Da)</th>
<th>Partition Coefficient $K_{ol/w}$</th>
<th>Passive Permeability $P$ (cm/hr)</th>
<th>Predicted $E$ Value</th>
<th>Experimentally Measured $E$ Value</th>
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</thead>
<tbody>
<tr>
<td>Dexemethasone</td>
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<td>1 MHz 1.5 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>392</td>
<td>97</td>
<td>$4.4 \times 10^{-4}$</td>
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<td>Significant Enhancement</td>
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<tr>
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<td>Human Skin In Vivo</td>
<td>1 MHz 2 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>300</td>
<td>$1.5 \times 10^{-4}$</td>
<td>12</td>
<td>Significant Enhancement</td>
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<tr>
<td>Hydrocortisone</td>
<td>Dog Skin In Vivo</td>
<td>1 MHz 1 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>362</td>
<td>40</td>
<td>$1.3 \times 10^{-4}$</td>
<td>3</td>
<td>Significant Enhancement</td>
</tr>
<tr>
<td></td>
<td>Human Skin In Vivo</td>
<td>1 MHz 2 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>362</td>
<td>40</td>
<td>$1.3 \times 10^{-4}$</td>
<td>3</td>
<td>Significant Enhancement</td>
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<tr>
<td></td>
<td>Human Skin In Vivo</td>
<td>1 MHz 3 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>362</td>
<td>40</td>
<td>$1.3 \times 10^{-4}$</td>
<td>3</td>
<td>Significant Enhancement</td>
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<td></td>
<td>Swine Skin In Vivo</td>
<td>1 MHz 1.5 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>40</td>
<td>$1.3 \times 10^{-4}$</td>
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<td></td>
<td>Swine Skin In Vivo</td>
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<td>362</td>
<td>40</td>
<td>$1.3 \times 10^{-4}$</td>
<td>3</td>
<td>Significant Enhancement</td>
</tr>
<tr>
<td></td>
<td>Pig Skin In Vivo</td>
<td>1 MHz 1.5 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>362</td>
<td>40</td>
<td>$1.3 \times 10^{-4}$</td>
<td>3</td>
<td>Significant Enhancement</td>
</tr>
<tr>
<td>Indomethacin</td>
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<td>No Enhancement</td>
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<td>Significant Enhancement</td>
</tr>
<tr>
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<td>Hairless Rat Skin In Vivo</td>
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<td>$4.4 \times 10^{-4}$</td>
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<td>Significant Enhancement</td>
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<td>No Significant Enhancement</td>
</tr>
<tr>
<td></td>
<td>Human Skin In Vivo</td>
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<td>138</td>
<td>0.1</td>
<td>$1 \times 10^{-4}$</td>
<td>0.05</td>
<td>No Significant Enhancement</td>
</tr>
<tr>
<td>Caffeeine</td>
<td>Human Skin In Vivo</td>
<td>1 MHz 2 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>194</td>
<td>1</td>
<td>$1.0 \times 10^{-4}$</td>
<td>0.3</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>Human Skin In Vivo</td>
<td>1 MHz 2 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>346</td>
<td>87</td>
<td>$3.0 \times 10^{-4}$</td>
<td>3</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>Estradiol</td>
<td>Human Skin In Vivo</td>
<td>1 MHz 2 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>272</td>
<td>7000</td>
<td>$3.2 \times 10^{-3}$</td>
<td>6</td>
<td>12.1 ± 1.5</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Human Skin In Vivo</td>
<td>1 MHz 2 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>274</td>
<td>6000</td>
<td>$1.3 \times 10^{-2}$</td>
<td>1</td>
<td>0.2 ± 0.5</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Human Skin In Vivo</td>
<td>1 MHz 2 W/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>288</td>
<td>2070</td>
<td>$2.2 \times 10^{-3}$</td>
<td>4</td>
<td>4.1 ± 1.1</td>
</tr>
</tbody>
</table>

Table 3: Summary of literature data on therapeutic sonophoresis.*

In Figure 14, the empty circles correspond to drugs for which no enhancement of transdermal delivery has been observed during sonophoresis, while filled circles correspond to drugs for which an experimentally observed enhancement has been reported [7, 9, 11, 17, 19, 22-26, 30-36, 38, 40-42, 45, 47, 48, 53, 83]. Note that most literature data on sonophoresis were reported in terms of the drug pharmacological activity in in vivo or clinical experiments (for example, induction of anesthesia in the case of lidocaine). Since an enhancement in the pharmacological activity cannot be easily related to the enhancement in permeability, this type

* For additional details on the data presented in Table 3, see Ref. [82].
of data offers only qualitative information regarding the drug permeability enhancements. In addition, some of these data were obtained using different animal models, and hence cannot be quantitatively compared with the model predictions, which were derived for human skin.

As can be seen from Figure 14, drugs having a predicted $E'$ value smaller than one exhibit no sonophoretic enhancement, while those having a predicted $E'$ value greater than one exhibit sonophoretic enhancement. Of particular interest are four drugs that have been commonly used for sonophoresis. These are hydrocortisone, indomethacin, lidocaine and salicylic acid. Equation [10] predicts that application of ultrasound should enhance transdermal delivery of hydrocortisone and indomethacin ($E'$>1) but not of lidocaine and salicylic acid ($E'$<1), a prediction that is in agreement with observations by various investigators [7, 9, 11, 12, 17-19, 22-26, 30, 33, 34, 36, 38, 40, 42, 43, 45, 47, 48, 51-53, 83]. Fundamentally, this suggests that the passive diffusion coefficients of hydrocortisone and indomethacin through the SC bilayers are very low compared to those through the disordered SC bilayers ($D^\infty/D_0$>1 in Eq. [8]) resulting in high $E'$ values. On the other hand, the passive diffusion coefficients of salicylic acid and lidocaine are comparable to those through the disorganized bilayer phase ($D^\infty/D_0 \sim 1$ in Eq. [8]) resulting in low values of $E'$. Therefore, Eq. [9] explains the variation of sonophoretic enhancement from drug to drug observed in the literature and offers an explanation for the controversy on this issue.

In summary, therapeutic ultrasound enhances transdermal transport of drugs by a factor of up to ~10, although the enhancement varies from drug to drug. Experimental analysis suggests that cavitation plays an important role in sonophoresis. Cavitation bubbles probably disorder the SC lipid bilayers, thereby enhancing transdermal transport. A theoretical model was developed to predict the sonophoretic transdermal transport enhancement. The theoretical predictions, as well as the measured sonophoretic enhancements for different permeants, suggest that the drugs passively diffusing through the skin at a slow rate are most enhanced by the application of ultrasound. These findings provide quantitative guidelines for estimating the efficacy of therapeutic sonophoresis in enhancing transdermal drug delivery. They also rationalize the variation of therapeutic sonophoretic efficiency from drug to drug reported by various investigators.

The next objective is to utilize the mechanistic understanding of therapeutic sonophoresis discussed in this chapter to increase the efficacy of sonophoresis. It appears logical that since cavitation effects are inversely proportional to ultrasound frequency, low-frequency ultrasound should enhance transdermal transport much more than that achieved using therapeutic ultrasound. To assess this hypothesis, sonophoresis experiments were performed at very low frequencies (20 KHz). Results of these experiments are discussed in the next chapter.
5. Low-Frequency Sonophoresis

5.1. Low-Frequency Sonophoresis In Vitro: The ultrasound conditions chosen for these studies involve a frequency of 20 KHz, an intensity in the range of 0-225 mW/cm², and a pulsed application of 100 msec pulses applied every second. A frequency of 20 KHz was chosen since it is the lowest possible ultrasound frequency. Sound at frequencies below 20 KHz is audible. Pulsed ultrasound application was chosen to minimize any thermal effects, although the choice of duty cycle was arbitrary. The chosen value of duty cycle limited the increase in skin temperature to less than 20°C. The intensities were chosen in the range of 0-225 mW/cm² as representative values. It should be noted that no values of the parameters chosen here, including intensity and duty cycle, are at optimal or limiting levels. A typical ultrasound condition used in the sonophoresis experiments correspond to a frequency of 20 KHz, a duty cycle of 10% (100 msec pulses applied every second), and an intensity of 125 mW/cm². Experimental details regarding low-frequency sonophoresis experiments are discussed in Chapter 3.

Application of low-frequency ultrasound (20 KHz, 125 mW/cm², 100 msec pulses applied every second) enhances transdermal transport across human cadaver skin in vitro for all the permeants examined, including estradiol, salicylic acid, corticosterone, aldosterone, sucrose, water, and butanol, by a factor in the range of 3 to 5000 [84]. As an example, Figure 15 shows the effect of ultrasound on the amount of salicylic acid transported (represented as the percent of the amount present in the donor compartment) across the skin (filled circles). The amount of salicylic acid transported by passive permeation, though detectable, is very small and is also shown (empty circles). The transdermal salicylic acid flux (proportional to the slope of the curves) increases with time and reaches a nearly steady-state value after 3 hours (indicated by the near constant slope of the curve shown in Figure 15). The sonophoretic skin permeability to salicylic acid was calculated using equations described in Chapter 3 and the experimental transdermal salicylic acid flux during the 4th and 5th hour, and was found to be 0.04 cm/hr. This sonophoretic permeability is about 400 times higher than the passive permeability of salicylic acid (1×10⁻⁴ cm/hr, measured in a different set of experiments). Application of ultrasound also enhances skin permeability to a variety of other permeants as shown in Table 4 below.
Figure 15- Percent salicylic acid transported across the skin in the presence of ultrasound (●) (20 KHz, 125 mW/cm², 100 msec pulses applied every second) as a function of time. Controls are shown by (O). Each number indicates mean ± SD (error bars) of 3 experiments.

<table>
<thead>
<tr>
<th>Permeant</th>
<th>Molecular Weight (Da)</th>
<th>Octanol-Water Partition Coefficient $K_{o/w}$</th>
<th>Passive Skin Permeability $PP$ (cm/hr)</th>
<th>Sonophoretic Permeability $P^{US}$ (cm/hr)</th>
<th>Enhancement Ratio $E = P^{US} / PP$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>360</td>
<td>12</td>
<td>$5.0 \times 10^{-5}$</td>
<td>0.070</td>
<td>1400</td>
</tr>
<tr>
<td>Butanol</td>
<td>74</td>
<td>7.5</td>
<td>$2.2 \times 10^{-3}$</td>
<td>0.064</td>
<td>29</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>346</td>
<td>87</td>
<td>$3.0 \times 10^{-4}$</td>
<td>0.024</td>
<td>80</td>
</tr>
<tr>
<td>Estradiol</td>
<td>272</td>
<td>7000</td>
<td>$3.0 \times 10^{-3}$</td>
<td>0.010</td>
<td>3</td>
</tr>
<tr>
<td>Salicylic Acid</td>
<td>138</td>
<td>0.1</td>
<td>$1.0 \times 10^{-4}$</td>
<td>0.040</td>
<td>400</td>
</tr>
<tr>
<td>Sucrose</td>
<td>342</td>
<td>0.0046</td>
<td>$5.2 \times 10^{-6}$</td>
<td>0.026</td>
<td>5000</td>
</tr>
<tr>
<td>Water</td>
<td>18</td>
<td>0.042</td>
<td>$3.0 \times 10^{-4}$</td>
<td>0.034</td>
<td>113</td>
</tr>
</tbody>
</table>

Table 4- Effect of low-frequency ultrasound on transdermal drug transport.

Transdermal transport enhancement induced by low-frequency ultrasound is much more significant than that induced by therapeutic ultrasound. Figure 16 compares the enhancement ratio (ratio of the sonophoretic and passive permeabilities measured in vitro across human cadaver skin) induced by therapeutic ultrasound (1MHz, 2 W/cm², continuous, indicated by empty bars(see also Table 2)) [9] and low-frequency ultrasound (20 KHz, 125 mW/cm², 100
msec pulses applied every second, indicated by hatched bars) (see also Table 4) for four permeants, butanol, corticosterone, salicylic acid, and sucrose.

![Bar chart showing log (enhancement ratio) for butanol, corticosterone, salicylic acid, and sucrose.]

**Figure 16**- Comparison of the sonophoretic enhancement ratio under therapeutic (white bars) and low-frequency (hatched bars) ultrasound conditions for butanol, corticosterone, salicylic acid, and sucrose.

Although the intensity is about 16-fold lower and the duty cycle is about 10-fold lower, low-frequency ultrasound enhances transdermal transport by as much as 1000-fold higher than that induced by therapeutic ultrasound. Another advantage of low-frequency sonophoresis as compared to therapeutic ultrasound is that the former can induce transdermal transport of drugs which do not passively permeate across the skin, while in many cases, the latter cannot. It is noteworthy that the sonophoretic permeabilities to all the permeants listed in Table 4 are nearly the same, that is, in the range of 0.01 cm/hr to 0.07 cm/hr, although their physico-chemical properties, including molecular weight and lipophilicity (represented in terms of the octanol-water partition coefficient, $K_{ow}$) are quite different. This behavior is different from that exhibited by the passive skin permeability which decreases dramatically with increasing permeant molecular weight and decreasing lipophilicity, thus allowing only a few drugs to permeate the skin at a therapeutically significant rate [85]. The least hydrophobic drug that is currently delivered transdermally for clinical applications is nicotine ($K_{ow} = 25$) and the largest drug that is delivered transdermally is fentanyl (MW = 336). The independence of the sonophoretic skin permeability on permeant characteristics suggests that low-frequency sonophoresis may potentially provide a method for the delivery of a wide variety of compounds irrespective of their physico-chemical properties. The observed sonophoretic drug permeability in the range of 0.01 cm/hr to 0.07 cm/hr may be sufficiently high to deliver therapeutic doses of many drugs since the passive skin permeability of all seven drugs that are
currently administered transdermally in clinical applications by passive transport is less than 0.02 cm/hr [85]. In addition, the sonophoretic permeability may be further increased by optimizing the ultrasound parameters, including intensity and pulse length.

5.2. Low-Frequency Sonophoresis In Vivo: In order to assess the efficacy of low-frequency ultrasound in enhancing transdermal transport across living skin, in vivo experiments were performed using hairless rats as an animal model. The transport properties of hairless rat and hairless mouse skin have been shown to resemble those of human skin [63, 86]. The passive permeability of the hairless rat skin to many compounds is within a factor of 2-5 of the human skin permeability. For a detailed discussion of the choice of animal model for transport experiments, see Ref. [86]. Salicylic acid was used as the model molecule for these studies since salicylates are one of the most important non-steroidal anti-inflammatory agents used for the treatment of joint disorders, such as arthritis. Transdermal delivery of salicylates provides an advantageous alternative to oral delivery or injections, since the former allows local delivery of salicylate, thus avoiding its high systemic concentrations which could be toxic to the body. Figure 17 shows the variation of the amount of salicylic acid excreted in urine (represented as the percent of the total applied dose) with time upon 1 hour of sonophoresis.

![Graph showing the variation of the amount of salicylic acid excreted in urine (in dpm) as a function of time.](image)

**Figure 17**: Amount of salicylic acid measured in the urine of hairless rats (in dpm) as a function of time. Each number indicates mean ± SD (error bars) of 3 experiments. Ultrasound was tuned ON at time 0 and turned OFF at time 1 hr.

About 16000 dpm (0.15% of the applied dose) of salicylic acid was found in the urine of rats exposed to ultrasound at the end of 2 hours. The amount in the urine of control rats was below the detection limit (50 dpm or 0.0005 % of the applied dose). This result suggests that application of low-frequency ultrasound enhances transdermal salicylic acid transport in vivo by
at least 300-fold, an enhancement comparable to the 400-fold enhancement measured in vitro across human skin.

5.3. Safety of Low-Frequency Sonophoresis: We evaluated, at a preliminary level, the safety issues associated with low-frequency sonophoresis. The safety of low-frequency sonophoresis involves two main issues: i) the reversibility of the skin barrier properties after turning ultrasound OFF, and ii) the effect of low-frequency ultrasound on the living parts of the skin and underlying tissues. Each issue is discussed in detail below.

Recovery of the Skin Barrier Properties after Sonophoresis: In order to assess the recovery of the barrier properties of the skin after short (1 hour) as well as long (5 hours) ultrasound exposures, skin pieces were exposed to ultrasound (20 KHz, 125 mW/cm², 100 msec pulses applied every second) for 1 hour in the diffusion cell with the donor and receiver compartments filled with PBS. 3H labeled water was then added in the donor compartment, and the transdermal flux of water was measured for up to 12 hours after ultrasound exposure. These experiments were repeated for a 5-hour ultrasound exposure using different skin pieces. In the case of a 1-hour long exposure, the skin permeability to water measured within 2 hours post-exposure was comparable to the passive skin permeability to water (see Table 5). In the case of a 5-hour long exposure, the skin permeability 2 hours post-exposure was about 6 times higher than the passive permeability to water (see Table 5). However, this value continued to decrease, and was within a factor of 2 of the passive skin water permeability 12 hours post-exposure. Hence, it appears that application of low-frequency ultrasound does not cause a long-term change in the skin barrier properties measured in terms of water permeability.

<table>
<thead>
<tr>
<th>Time</th>
<th>Post-Exposure Skin Water Permeability Normalized by the Passive Water Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Hour Exposure</td>
</tr>
<tr>
<td>After Exposure</td>
<td>11±4</td>
</tr>
<tr>
<td>2 Hours Post-Exposure</td>
<td>1.2±0.8</td>
</tr>
<tr>
<td>12 Hours Post-Exposure</td>
<td>1.3±0.9</td>
</tr>
<tr>
<td></td>
<td>5 Hour Exposure</td>
</tr>
<tr>
<td></td>
<td>112±41</td>
</tr>
<tr>
<td>2 Hours Post-Exposure</td>
<td>6.2±3.2</td>
</tr>
<tr>
<td>12 Hours Post-Exposure</td>
<td>2.1±1.2</td>
</tr>
</tbody>
</table>

Table 5-Recovery of skin barrier properties after sonophoresis.

Biological Effects of Low-Frequency Ultrasound: Low-frequency ultrasound (frequency in the range of 20-85 KHz) is currently used by dentists for tooth cleaning [62]. In view of this, significant efforts have been devoted to investigate probable biological effects of low-frequency ultrasound
[56, 71]. However, no conclusions have been reached regarding the limiting ultrasound conditions required to ensure safe exposure.

In order to assess the effect of low-frequency ultrasound on living skin cells, initial histological studies were performed using hairless rats as an animal model. Figure 18a shows hairless rat skin not exposed to ultrasound (control). Figure 18b shows hairless rat skin exposed to ultrasound (20 KHz, 100 msec pulses applied every second, 62.5 mW/cm²). Figure 18C shows hairless rat skin exposed to ultrasound at an intensity of 125 mW/cm² (20 KHz, 100 msec pulses applied every second) and Figure 18D shows hairless rat skin exposed to ultrasound at 225 mW/cm² (20 KHz, 100 msec pulses applied every second).

Figure 18a - Histology of hairless rat skin not exposed to ultrasound. Figure 18b - Histology of hairless rat skin exposed to ultrasound (20 KHz, 62 mW/cm², 100 msec pulses applied every second). Figure 18c - Histology of hairless rat skin exposed to ultrasound (20 KHz, 125 mW/cm², 100 msec pulses applied every second). Figure 18d - Histology of hairless rat skin exposed to ultrasound (20 KHz, 225 mW/cm², 100 msec pulses applied every second).
The histological studies indicated no physical damage in the skin or in the underlying muscle tissues exposed to ultrasound at all the intensities examined in the experiments described above. Furthermore, the regions of hairless rat epidermis exposed to ultrasound were intact and showed no signs of abnormality.

Although further research (discussed in Chapter 8) focusing on safety issues is required before arriving at any final conclusions regarding the safety of low-frequency ultrasound, these preliminary studies indicate that low-frequency ultrasound does not induce damage to the skin and underlying tissues.

In summary, low-frequency ultrasound enhances transdermal transport up to 1000-fold higher than that induced by therapeutic ultrasound. Furthermore, application of low-frequency ultrasound does not seem to induce any long-term damage to the skin barrier properties and underlying tissues. In the next chapter, the mechanism by which low-frequency induces such dramatic enhancements of transdermal transport is discussed.
6. Mechanism of Low-Frequency Sonophoresis

6.1. Role of cavitation in Low-Frequency Sonophoresis: As described in Chapter 4, cavitation plays a major role in sonophoresis performed using therapeutic ultrasound (frequency in the range of 1 MHz - 3 MHz, and intensity in the range of 0 - 2 W/cm²). Specifically, oscillations of cavitation bubbles induce disorder in the SC lipid bilayers, thereby enhancing the transport of drugs across the SC [9]. Since it is known that cavitation effects in fluids vary inversely with ultrasound frequency [69], cavitation effects should play an even more important role in low-frequency sonophoresis. The important question is why does cavitation under low-frequency ultrasound conditions induces a dramatic enhancement of transdermal transport as described in Chapter 5.

Cavitation may occur inside as well as outside the skin upon ultrasound exposure. Cavitation at either location may enhance transdermal transport in two ways: (i) cavitation may induce convective transport across the skin, and (ii) oscillations of cavitation bubbles may disorganize the SC lipid bilayers. Experiments were performed to assess the importance of (i) and (ii) in low-frequency sonophoresis.

(i) Role of Cavitation-Induced Convection in Sonophoresis: Oscillations of cavitation bubbles may induce convective velocities, a phenomenon referred to as microstreaming [57]. If such velocities are generated inside or near the skin, they may induce convective transport across the skin. In order to assess the importance of convection during sonophoresis, passive as well as sonophoretic permeability of delipidized SC to water was measured. Water was chosen as a model permeant for these studies since water would be the primary fluid involved in the possible convection across the skin. It was anticipated that, since probably there were no bilayers present in the delipidized skin, if a sonophoretic enhancement of water transport across the delipidized skin is observed, it should be due to convection and not due to bilayer disordering. This way, one could isolate the contributions of possible convective transport and bilayer disordering during sonophoresis. The SC was first separated from the epidermis by soaking the heat-stripped human cadaver epidermis in trypsin [87]. The lipids in the SC were then removed by soaking it in a mixture of chloroform/methanol (2:1) [88]. After 3 days of soaking, the SC was washed with ethanol and water. The passive permeability of the delipidized skin to water was found to be about 8 cm/hr (compared to $3 \times 10^{-4}$ cm/hr of the
normal skin). This observation shows that keratinocytes do not offer significant resistance to transdermal water transport. Upon application of ultrasound (20 KHz, 125 mW/cm², 100 msec pulses applied every second), no enhancement of water transport across the delipidized SC was observed, suggesting that cavitation-induced convection may not play a major role in low-frequency sonophoresis.

(ii) **Role of Cavitation-Induced Bilayer Disordering in Sonophoresis:** To assess the role of bilayer disordering in low-frequency sonophoresis, electrical resistance measurements of the skin during sonophoresis were performed.

The electrical resistance of the skin is a good, instantaneous indicator of the structural properties of the skin [66]. Under normal conditions, ionic transport through the skin occurs partly through the follicles and partly through the intercellular bilayers [75]. Due to significant hindrance of the ionic transport through the bilayers, the electrical resistance of the skin is very high. If application of ultrasound disorders the SC lipid bilayers, a decrease in the electrical resistance of the skin should be observed due to reduced hindrance. In addition, if significant bilayer disordering occurs, ions could transport across the lipid domains and keratinocytes, a phenomenon which would reduce the skin resistance by more than 10-fold [75]. Figure 19 shows the measured variation of the skin electrical resistance (normalized by the resistance in the absence of ultrasound) with time upon ultrasound application (20 KHz, 125 mW/cm², 100 msec pulses applied every second).

![Graph](image)

**Figure 19:** Variation of the normalized skin electrical resistance in the presence of ultrasound (20 KHz, 125 mW/cm², 100 msec pulses applied every second) with time. Each number indicates mean ± SD (error bars) of 3 experiments.
The observed decrease in the skin electrical resistance suggests that low-frequency ultrasound induces disorder in the SC lipid bilayers. Moreover, the 25-fold decrease in the skin electrical resistance suggests that application of low-frequency ultrasound induces ionic transport across the lipid domains and keratinocytes. The occurrence of trans-keratinocyte pathways during low-frequency sonophoresis is also supported by the observed characteristic dependence of the sonophoretic permeability on the molecular properties of the permeants as explained below.

6.2. Mechanism of Low-Frequency Sonophoresis: The passive transdermal transport of drugs occurs mostly through the lipid bilayers of the SC [2]. Accordingly, the passive skin permeability is proportional to the lipid partition coefficient of the drug [75] (see also Eq. [2] in Chapter 4). On the other hand, the sonophoretic permeability under low-frequency ultrasound conditions is not proportional to the lipophilicity of drugs. In fact, the sonophoretic skin permeabilities of the seven permeants examined in this study, including water, sucrose, estradiol, corticosterone, aldosterone, salicylic acid, and butanol, are remarkably similar although their lipophilicities (as reflected by their octanol-water partition coefficients, $K_{o/w}$) differ by five orders of magnitude (Table 4). This lack of correlation between the sonophoretic skin permeability and the permeant lipophilicity suggests that transdermal transport during low-frequency sonophoresis no longer occurs through the intercellular lipids. Instead, it occurs through aqueous pathways across the SC. These aqueous pathways or channels may be generated due to the vigorous cavitation activity that may occur inside as well as outside the skin upon ultrasound application. Specifically, cavitation activity may disorganize the SC lipid bilayers followed by water penetration from the keratinocytes, leading to the formation of aqueous channels. Transdermal transport of permeants may then occur through these channels (Figure 20). The occurrence of transdermal transport through aqueous channels across the disordered lipid regions may enhance transdermal transport as compared to passive transport because: i) the diffusion coefficients of permeants through water, which is likely to primarily occupy the channels generated by ultrasound, are up to 1000-fold higher than those through the ordered lipid bilayers. Specifically, the diffusion coefficients of all the permeants listed in Table 4 in water are about $1 \times 10^{-5} \text{ cm}^2/\text{s}$ [80] while their passive diffusion coefficients through the SC vary between $1 \times 10^{-6} \text{ cm}^2/\text{s}$ and $1 \times 10^{-9} \text{ cm}^2/\text{s}$ [75], and ii) the transport path length of these aqueous channels may be much shorter (by a factor of up to 25 [75]) than that through the tortuous intercellular lipids in the case of passive transport. Note that under conditions where aqueous channels are formed through the disordered lipid regions, transdermal transport is unlikely to occur through the intercellular route since the intercellular lipids may lose continuity due to the formation of the aqueous channels.
Based on the discussion presented above, the proposed mechanism of low-frequency sonophoresis is summarized as follows. Application of low-frequency ultrasound may induce cavitation inside as well as outside the skin. Cavitation occurring at either location may cause disordering of the SC lipids. In addition, oscillations of cavitation bubbles may result in significant water penetration into the disordered lipid regions. This may cause the formation of aqueous channels through the intercellular lipids of the SC (Figure 20). This allows permeants to transport across the disordered lipid domains as well as across the keratinocytes, thereby leading to enhanced transdermal transport.

Further support for this mechanism was provided by developing a mathematical model which is discussed next.

6.3. Mathematical Modeling of Low-Frequency Sonophoresis: As shown in Figure 20b, transdermal drug transport during low-frequency sonophoresis is assumed to occur through the aqueous channels across the disordered intercellular lipid regions. Since transport across keratinocytes is relatively fast, transport across the disordered intercellular lipid domains constitutes the rate limiting step in skin permeation during low-frequency sonophoresis. Note that the SC consists of several layers of keratinocytes (denoted as N, with N typically =15 [5]), suggesting that a permeant molecule has to cross about 15 intercellular lipid regions in order to transport across the SC. Consider a layer of intercellular lipid domain parallel to the skin surface (Figure 20b). Let $f$ be the average fractional area occupied by the aqueous channels in the plane.
of the lipid domain across which drugs may diffuse. Since these channels are filled with saline, it may be assumed that small molecules (MW < 500) diffuse through these channels with a diffusivity, $D_w$, equal to that through water (typically $1 \times 10^{-5}$ cm$^2$/s in the case of permeants having molecular weights of less than 500 [80] which are of main interest here).

Based on the mechanistic explanation discussed above, the sonophoretic skin permeability, $P^{us}$, to small molecules may be expressed as [75]:

$$P^{us} = \frac{K \phi D_w}{N \ell} \quad (\text{cm/h})$$  \[11\]

where $K$ is the permeant partition coefficient in the aqueous channels (may be assumed to be unity since the donor compartment of the diffusion cell as well as the transport channels are likely to be filled with the same saline medium), $N$ is the number of intercellular lipid regions that a molecule has to cross (typically 15 [75]), and $\ell$ is the thickness of each lipid region (typically 50 nm [75]) (Figure 20b). The parameters $\phi$, $D_w$, $N$, and $\ell$ in Eq. [11] are all independent of the permeant characteristics, thus suggesting that the sonophoretic permeability should be independent of the permeant characteristics. This prediction is consistent with the observations that the sonophoretic skin permeabilities to all seven permeants studied are similar, although their physico-chemical properties are significantly different (Table 4).

Similarly, the electrical conductivity of the skin during sonophoresis, $\sigma^{us}$ (related to the skin resistivity, $\rho^{us}$, by the relation, $\sigma^{us} = h/\rho^{us}$, where $h$ is the SC thickness) may be expressed as follows [75]:

$$\sigma^{us} = \frac{\phi \sigma_w}{N} \quad (\Omega \text{cm})^{-1}$$  \[12\]

where $\sigma_w$ is the electrical conductivity of saline ($1.2 \times 10^{-2}$ (Ωcm)$^{-1}$) [75]). Equations [11] and [12] relate the sonophoretic skin properties to fundamental transport properties and skin geometry. In these equations, $\phi$ is the only unknown parameter that needs to be determined experimentally. Note that $\phi$ depends on ultrasound parameters, including intensity, pulse length, and exposure time, and can be estimated from Eq. [12] by utilizing the experimentally measured values of $\sigma^{us}$. The skin electrical resistance, $R^{us}$, during the 4th and 5th hour of ultrasound (20 KHz, 125 mV/cm$^2$, 100 msec pulses applied every second) exposure was about 5100±3100Ω (about 4% of the electrical skin resistance in the absence of ultrasound (Figure 19)). This sonophoretic skin electrical resistance corresponds to $\overline{\rho}^{us} = 16000 \pm 9600 \Omega \cdot \text{cm}^2$ ($\overline{\rho}^{us} = \Lambda R^{us}$, where $\Lambda$ is the skin area = 3.14 cm$^2$), which, in turn, yields $\overline{\sigma}^{us} = 9 \times 10^{-8}$ (±60%) (Ωcm)$^{-1}$ ($\overline{\sigma}^{us} = h/\overline{\rho}^{us}$, where $h = 15 \times 10^{-4}$ cm). Substituting this value of $\overline{\sigma}^{us}$ in Eq. [12] yields $\phi = 1.2 \times 10^{-4}$ (±60%). This suggests that about 0.012% of the skin area is available for sonophoretic transport under these ultrasound conditions. After substituting $\phi = 1.2 \times 10^{-4}$ in Eq. [11], with
\[ D_W = 1 \times 10^{-5} \text{cm}^2/\text{s}, \quad N = 15, \quad \text{and} \quad \ell = 50 \times 10^{-7} \text{cm}, \quad \text{and expressing} \quad P^{\mu s} \quad \text{in units of cm/hr, one obtains:} \]

\[
P^{\mu s} = \left[ \frac{0.00012(1 \times 10^{-5})}{15(50 \times 10^{-7})} \right] \times 3600 = 0.057 \quad \text{(cm/hr)} \tag{13} \]

Therefore, the model predicts that the sonophoretic permeability of all low-molecular weight drugs (MW < 500) under the ultrasound conditions examined here (20 KHz, 125 mW/cm², 100 msec pulses applied every second) is about 0.057 (±60%) cm/hr, that is, in the range of 0.023 cm/hr to 0.09 cm/hr. This value is in reasonable agreement with the experimentally measured sonophoretic permeabilities of several permeants listed in Table 4, which are in the range of 0.01 cm/hr for estradiol to 0.07 cm/hr for aldosterone. The sonophoretic enhancement, \( E \), defined as the ratio of the sonophoretic permeability, \( P^{\mu s} \), and the passive permeability, \( P^p \), may then be expressed as follows:

\[
E = \frac{0.057}{P^p} \tag{14} \]

Figure 21 compares the limiting values of the sonophoretic enhancements predicted by Eq. [14] with the experimentally observed enhancements for seven permanents as reported in Table 4. The lines correspond to the upper (\( P^{\mu s} = 0.09 \text{cm/hr} \)) and lower (\( P^{\mu s} = 0.023 \text{cm/hr} \)) limits of the predicted sonophoretic permeabilities. Figure 21 indicates that the predicted values of the sonophoretic enhancements are in reasonable agreement with the experimental values.

This analysis of low-frequency sonophoresis explains why low-frequency ultrasound can induce transdermal transport of drugs which exhibit very low passive transdermal transport. Drugs possessing low passive permeabilities are either: i) hydrophilic, which makes their partitioning into the SC bilayers difficult, or ii) large in molecular size (for example, aldosterone), which reduces their diffusion coefficients in the SC. Low-frequency ultrasound overcomes both of these limitations by providing aqueous transport channels across the skin. Since these channels are filled with saline, hydrophilic drugs can easily partition into the SC. In addition, diffusion of drugs through water is much faster than that through ordered lipid bilayer regions, thus allowing drugs to transport across the skin at a faster rate. Therefore, molecules such as hydrophilic drugs, may permeate skin with relative ease in the presence of low-frequency ultrasound. On the other hand, transdermal transport of lipophilic drugs may not be enhanced significantly by low-frequency ultrasound. This occurs since the partition coefficients of lipophilic drugs into the aqueous channels are likely to be lower than those into the lipid domains. As a result, the increase in the permeant diffusion coefficient in the SC due
to channel formation may be partly offset for by the reduction in the partition coefficient. Hence the sonophoretic enhancement is likely to be less significant for hydrophobic drugs compared to that for hydrophilic drugs. Indeed, it is observed that while low-frequency ultrasound enhances transdermal transport of aldosterone ($K_{0/w} = 17$) by a factor of 1400, it enhances transdermal transport of estradiol ($K_{0/w} = 7000$) only by a factor of 3, although both drugs possess similar molecular weights.

![Graph showing sonophoretic enhancements vs. passive permeability](image)

**Figure 21** - Comparison of the sonophoretic enhancements, $E$, predicted using Eq. [14] with the experimentally observed sonophoretic enhancements (Table 4). The predicted $E$ values are shown by two lines as the upper ($P^{US} = 0.09$ cm/hr) and lower ($P^{US} = 0.023$ cm/hr) limits on the predictions. The filled circles indicate experimentally measured $E$ values. Each number indicates mean ± SD (error bars) of 3 experiments.

6.4. **Summary of Mathematical Modeling of Sonophoresis**: For the sake of comparison, equations to predict the transdermal transport enhancement in the presence of therapeutic and low-frequency ultrasound are summarized in Table 6. The general equation to predict sonophoretic enhancement under therapeutic ultrasound is obtained by substituting Eq. [2] and the correlation $K_m = K_{0/w}^{0.75}$ in Eq. [8]. The fraction of bilayers disordered by therapeutic ultrasound, $f$, is represented as a function of ultrasound frequency, $f_r$, intensity, $I$, and duty cycle, $d$.

The general equation for low-frequency sonophoresis is obtained from Eq. [10] after substituting values of $N$, $D_{ww}$, and $\epsilon$ as mentioned earlier. The area fraction of aqueous channels, $\phi$, is represented as a function of ultrasound frequency, $f_r$, intensity, $I$, and duty cycle, $d$. Equations under typical conditions are derived by substituting appropriate values of $f$ (0.13) and $\phi$ (0.0012) in the corresponding equations.
In the equations presented in Table 6, $E$ is the sonophoretic enhancement of skin permeability, $P^P$ is the passive skin permeability (cm/hr), $K_{0/w}$ is the octanol-water partition coefficient, $f$ is the fraction of bilayers disordered by the application of therapeutic ultrasound, and $\phi$ is the fraction of skin area occupied by the aqueous channels in low-frequency sonophoresis.

<table>
<thead>
<tr>
<th></th>
<th><strong>Therapeutic Ultrasound</strong></th>
<th><strong>Low-Frequency Ultrasound</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General Equations</strong></td>
<td>$E = (1 - f(fr,l,d)) + \frac{f(fr,l,d)K_{0/w}^{0.75}}{5.2 \times 10^5 P^p}$</td>
<td>$E = \frac{480\phi(fr,l,d)}{P^p}$</td>
</tr>
<tr>
<td>(Obtained from Eq. [2], $K_m = K_{0/w}^{0.75}$, and Eq. [8])</td>
<td>(Obtained from Eq. [13] and Eq. [14])</td>
<td></td>
</tr>
<tr>
<td><strong>Typical Conditions</strong></td>
<td>1 MHz, 2 W/cm$^2$, Continuous</td>
<td>20 KHz, 125 mW/cm$^2$, 10 % Duty Cycle</td>
</tr>
<tr>
<td><strong>Equations Under Typical Conditions</strong></td>
<td>$E = 0.87 + \frac{K_{0/w}^{0.75}}{4 \times 10^5 P^p}$</td>
<td>$E = \frac{0.057}{P^p}$</td>
</tr>
<tr>
<td>(Equation [11])</td>
<td>(Equation [14])</td>
<td></td>
</tr>
</tbody>
</table>

Table 6—Comparison of the model equations to predict therapeutic and low-frequency sonophoresis.

In summary, the skin drug permeability in the presence of low-frequency ultrasound is nearly independent of the molecular weight and lipophilicity of the drug. It is suggested that application of low-frequency ultrasound generates aqueous channels across the SC, through which transdermal transport of drugs may occur. This, in turn, results in a similar sonophoretic permeability of various drugs is despite their different physico-chemical characteristics. A quantitative model was developed to predict the effect of low-frequency ultrasound on transdermal transport of drugs. The predictions of the model are in reasonable quantitative agreement with experimental observations.

It is conceivable that since low-frequency ultrasound enhances transdermal drug transport of low-molecular weight drugs, it could enhance transdermal transport of high-molecular weight proteins. This issue is discussed in the next chapter.
7. Transdermal Protein Delivery

The transdermal route of drug administration could be advantageous in the delivery of many therapeutic proteins, because: (i) proteins are susceptible to gastro-intestinal degradation and exhibit poor gastro-intestinal uptake, (ii) proteins such as interferons are cleared rapidly from the blood, and need to be delivered at a sustained rate in order to maintain their blood-concentration at a high value, and (iii) transdermal devices are easier to use than injections. However, the passive skin permeability to high-molecular weight proteins is essentially zero, thus prohibiting transdermal delivery of proteins. To assess whether application of ultrasound enhances transdermal protein flux, experiments were performed using three proteins, insulin, γ-interferon, and erythropoeitin. Some information on these proteins is provided below.

7.1. Model Proteins: Three model proteins, insulin, γ-interferon, and erythropoeitin, were used to investigate sonophoretic transdermal protein delivery. These proteins were chosen because of their relevance for therapeutic applications, as well as because of their different molecular sizes which would allow us to understand the dependence of sonophoretic permeability on molecular size.

Insulin is a 6,000 Da protein given for the treatment of diabetes. It is commercially available in injectable forms at concentrations in the range of 100 U/ml and 500 U/ml (Eli Lilly). High-concentration formulations (10000 U/ml) are also available in certain cases. Currently, there are about 1.5 million diabetics worldwide of which about half are treated by insulin administration. A typical diabetic patient takes about 0.5 U/Kg dose of insulin three times a day. 1 U of insulin corresponds to about 40 μg of insulin. For a thorough review of insulin production, structure, formulations, and pharmaceutical developments, see Ref. [89].

γ-interferon is a 17,000 Da protein which is given to enhance the immune response of patients suffering from AIDS, cancer or any viral infection, although there are no well-established clinical applications of γ-interferon [90]. A typical dose of γ-interferon taken by a patient suffering from viral disease is about 5×10⁶ U/day. 1 U of γ-interferon corresponds to about 1 pg. For a review of the structure and applications of γ-interferon, see Refs. [91, 92].

Erythropoeitin is a 48,000 Da glycoprotein given to enhance erythropoiesis in patients suffering from severe anemia [93]. It is available in injectable forms at concentrations in the
range of 200 U/ml to 1000 U/ml (Amgen). A typical dose taken by an anemic patient corresponds to about 400U/day [94]. 1 U of erythropoietin weighs about 7.6 ng. More information on the structure, development, and applications of erythropoietin can be found in Refs. [93, 94].

An analysis of sonophoretic transdermal protein transport was performed [95] with three objectives; i) to assess the efficacy of ultrasound in enhancing transdermal protein transport in vitro across human cadaver skin, ii) to assess the efficacy of ultrasound in enhancing transdermal protein transport in vivo across hairless rat skin, and iii) to assess the safety issues associated with sonophoresis in vitro as well as in vivo.

Each of these objectives is discussed below.

7.2. Protein Sonophoresis In Vitro: Skin permeability to the above mentioned proteins was measured in the presence of ultrasound in vitro across human cadaver epidermis according to the experimental procedures described in Chapter 3. Ultrasound application induced significant transdermal permeation of insulin, \( \gamma \)-interferon, and erythropoietin. For example, Figure 22 shows the amount of insulin transported across human cadaver skin (in vitro) at different times in the presence of ultrasound (20 KHz, 100 msec pulses applied every second) at various intensities (■ -12.5 mW/cm\(^2\), ○ - 62.5 mW/cm\(^2\), ● -125 mW/cm\(^2\), and ▲ - 225 mW/cm\(^2\)).

The transdermal insulin transport is proportional to the ultrasound intensity. This proportionality can be clearly seen in Figure 23, where skin insulin permeability calculated from transdermal insulin flux during the first hour of sonophoresis (Figure 22) is plotted as a function of ultrasound intensity (see Chapter 3 for a discussion of calculations of permeability). The sonophoretic permeability varies nearly exponentially with ultrasound intensity, probably due to a highly non-linear dependence of cavitation on ultrasound intensity. Accordingly, ultrasound intensity may be potentially used to control transdermal insulin delivery.

As can be seen from Figure 23, the human skin permeability to insulin calculated from the insulin flux during the first hour at an ultrasound intensity of 225 mW/cm\(^2\) is \(3.3 \times 10^{-3}\) (±35%) cm/h. The permeability to \( \gamma \)-interferon was measured under similar conditions and was found to be \(8 \times 10^{-4}\) (± 22%) cm/h, and that to erythropoietin was found to be \(9.8 \times 10^{-6}\) (± 40%) cm/h. With these skin permeabilities, it may be possible to deliver these proteins transdermally at a therapeutically relevant rate. For example, one could deliver an insulin dose of about 12 U/h (a dose given three times a day to a diabetic patient[89]) from a transdermal patch having an area of 40 cm\(^2\) (an area comparable to that of a fentanyl patch made by ALZA corporation) containing insulin at a concentration of 100 U/ml. In other words, one hour of sonophoresis performed three times a day could deliver the required daily dose of insulin to a diabetic
patient. Similarly, a γ-interferon dose of about $5 \times 10^6$ U/h (a daily dose required to enhance the immune response of patients suffering from viral infection or cancer [90]), and an erythropoietin dose of about 140 U/h (a dose that may be given three times a day to patients suffering from severe anemia [93]) may be delivered from a similar patch by application of ultrasound.

![Graph 1](image1.png)  
**Figure 22**  
Time variation of the amount of insulin transported across human cadaver skin (*in vitro*) in the presence of ultrasound (20 KHz, 100 msec pulses applied every second) at various intensities ( ■ - 12.5 mW/cm$^2$, ● - 62.5 mW/cm$^2$, ○ - 125 mW/cm$^2$, and ▲ - 225 mW/cm$^2$). (n=3-4, error bars indicate SD(Standard Deviation)).

Figure 23- Variation of the transdermal insulin permeability (*in vitro*) with ultrasound intensity (20 KHz, 100 msec pulses applied every second). (n=3-4, error bars indicate SD.) The skin is impermeable to insulin at an ultrasound intensity = 0.

The sonophoretic enhancement of transdermal protein transport was further analyzed in vivo using insulin as a model protein.

7.3 Protein Sonophoresis In Vivo: To assess the efficacy of ultrasound in enhancing transdermal flux in an *in vivo* model, insulin sonophoresis experiments were performed on hairless rats. Figure 24 shows the blood glucose level of hairless rats upon a 1 hour insulin-ultrasound treatment (20 KHz, 100 msec pulses applied every second at intensities of 0 mW/cm$^2$ (□), 12.5 mW/cm$^2$(●), 62.5 mW/cm$^2$ (▲), 125 mW/cm$^2$ (●), and 225 mW/cm$^2$ (■)). An intensity-dependent decrease in the blood glucose level was observed upon ultrasound.
Figure 24 - Time variation of blood glucose levels of hairless rats upon 1 hour insulin-ultrasound treatment (ultrasound turned ON at 1 hour and turned OFF at 2 hours) at four different intensities (□ - Control (n=4), ● - 12.5 mW/cm² (n=3), ▲ - 62.5 mW/cm² (n=3), ○ - 125 mW/cm² (n=3), ■ - 225 mW/cm² (n=5)).

Figure 25 - Comparison of the blood glucose level of hairless rats treated for 1 hour (from time 1 to 2 hour) with sonophoresis at two intensities (▲ - 62.5 mW/cm² (n=3), ■ - 225 mW/cm²(n=5)) and those treated with a single subcutaneous injection at time 1 hour (dashed line - 1U (n=3), dotted line - 100 mU (n=3). A typical rat weighed about 400 g. The control is indicated by (N) (error bars (SD) shown on one set of data for subcutaneous as well as sonophoresis data).

Figure 26 - Time variation of blood glucose levels of hairless rats exposed to ultrasound (20 KHz, 225 mW/cm², 100 msec pulses applied every second) for different exposure times. Ultrasound was turned ON at 1 hour (indicated by the arrow) and was turned OFF after 1 minute (O) (n=3), 10 minutes (▲) (n=3), and 1 hour (■) (n=5). The control is indicated by (□). (error bars (SD) shown on one set of data).

Figure 27 - Time variation of blood glucose levels of diabetic hairless rats upon a 30 minute insulin-ultrasound treatment (ultrasound turned ON at 0.5 hr and turned OFF at 1 hr). (O - Diabetic Rats, ▲ - Normal Rats, ▲ - Diabetic Rats with insulin-ultrasound treatment.) (n=4 per experiment, error bars indicate SD).
application, indicating that low-frequency sonophoresis can effectively deliver intensity-dependent insulin doses across hairless rat skin.

To estimate the amount of insulin penetrating the hairless rat skin during sonophoresis at the various intensities reported in Figure 24, various known amounts of insulin in the range of 0 to 1 U were injected subcutaneously (most commonly used method of insulin administration today) in normal rats. The blood glucose levels of these rats were then compared with those of the normal rats undergoing sonophoresis. Subcutaneous injection of 100 mU and 1 U of insulin induced a decrease in the blood glucose level similar to that induced by sonophoresis using intensities of 62.5 mW/cm² and 225 mW/cm² respectively (see Figure 25). These results suggest that sonophoresis delivers intensity dependent insulin doses approximately in the range of 0 to 1 U transdermally (through an area of about 3 cm²).

In order to estimate the dependence of the amount of insulin delivered on ultrasound exposure time (in vivo), insulin-sonophoresis experiments were performed (20 KHz, 225 W/cm², 100 msec pulses applied every second) on normal rats for different exposure times in the range of 1 minute to 1 hour. Figure 26 shows that while a 1 hour exposure (■) decreases blood glucose level from about 250 mg/dl to about 30 mg/dl, a 10 minute exposure to ultrasound (▲) reduces the blood glucose level of hairless rats from about 250 to about 150 mg/dl. This result, when compared with the data reported in Figure 25, suggests that while a 1 hour ultrasound exposure delivers about 1 U of insulin, a 10 minute ultrasound application (225 mW/cm², 100 msec pulses applied every second) delivers about 100 mU through an area of 3 cm².

Additional experiments were performed to assess whether application of ultrasound can induce sufficient insulin transport across the skin of a diabetic hairless rat so that its blood glucose level becomes comparable to that of normal hairless rats. Figure 27 shows blood glucose levels of diabetic rats during ultrasound-insulin treatment. Insulin-ultrasound treatment (20 KHz, 225 mW/cm², 100 msec pulses applied every second) reduces the blood glucose level of diabetic hairless rats from about 400 mg/dl (the blood glucose level of diabetic rats) to 200 mg/dl (the blood glucose level of normal rats) in 30 minutes. To ensure that these changes in blood glucose level are accompanied by appropriate changes in plasma insulin concentrations, insulin concentrations in the blood of hairless rats were measured during sonophoresis. Normal hairless rats possessed a plasma insulin level of 101±31 picomolar while diabetic hairless rats possessed a value below our detection limit (34 picomolar). During sonophoresis, the levels of transdermally delivered human insulin in rat plasma reached a value of 77 (±28) picomolar after 30 minutes and a value of 178 (±84) picomolar after 1 hour. No significant change in the plasma concentration of indigenous rat insulin was observed during sonophoresis.
These results indicate that low-frequency sonophoresis can enhance transport of insulin across hairless rat skin in vivo. The safety of low-frequency ultrasound as a transdermal transport enhancer was evaluated next.

7.4 Safety of Low-Frequency Sonophoresis: As mentioned earlier, the safety of low-frequency sonophoresis involves two main issues: i) the reversibility of the skin barrier properties after turning ultrasound OFF, and ii) the effect of low-frequency ultrasound on the living parts of the skin and underlying tissues. Each issue is discussed below.

Recovery of the Skin Barrier Properties after Sonophoresis: Application of ultrasound under the above mentioned conditions did not appear to cause any permanent loss of the barrier properties of the skin. The transdermal insulin flux (proportional to the slope of the curves shown in Figure 22) three hours after turning ultrasound OFF was statistically insignificant. Additional studies on barrier recovery after sonophoresis were performed using water as a permeant. These studies were described in detail in Chapter 6. It was found that during sonophoresis, a water permeability enhancement of 100-fold was observed, of which about 94 (±3) % was recovered within 2 hours after turning ultrasound OFF, and 98 (±1) % was recovered within 15 hours. These results suggest that application of ultrasound does not induce any long-lasting loss of the skin barrier properties (see also Table 5 in Chapter 6).

Biological Effects of Low-Frequency Ultrasound: As described in Chapter 6, initial histological studies were performed using hairless rats as an animal model. Figure 18a shows hairless rat skin unexposed to ultrasound (control). Figure 18b shows hairless rat skin exposed to ultrasound (20 KHz, 100 msec pulses applied every second, 125 mW/cm²) and Figure 18c shows hairless rat skin exposed to ultrasound at an intensity of 225 mW/cm² (20 KHz, 100 msec pulses applied every second). The histological studies indicated no physical damage in the skin or in the underlying muscle tissues exposed to ultrasound at all the intensities examined in the experiments described above. Furthermore, the regions of hairless rat epidermis exposed to ultrasound were intact and showed no signs of abnormality. Although further research focusing on safety issues is required before arriving at any final conclusions regarding the safety of low-frequency ultrasound, these preliminary studies indicate that low-frequency ultrasound does not induce damage to the skin and underlying tissues.

The results described in this chapter indicate that low-frequency sonophoresis offers a potential method for transdermal protein administration. Its efficacy in enhancing the transdermal transport of high-molecular weight proteins, for example, insulin, makes it a potential non-invasive substitute for injections. Two different types of applications can be
imagined for sonophoretic transdermal drug delivery. First, a sonicator may be located in the
doctor's office or hospitals which can be used for the delivery of drugs. These devices could also
be owned by patients and kept at home for delivery of drugs. These devices can be operated on
an AC power supply, and could be used in the present form if required. A second type of
sonicator involves portable devices that patients can carry and use as required. Although
current low-frequency ultrasound devices are relatively large, one could imagine small pocket-
size sonicators being manufactured some day. Such devices operating on batteries have already
been manufactured for various medical applications including iontophoretic and
electroporative transdermal drug delivery in laboratories, and for ultrasound tooth brushes on a
commercial scale (Sonex International Corporation). Whether similar devices for sonophoretic
transdermal drug delivery can be powered by a battery, remains to be seen based on the
required electrical power. However, since a sonicator operating at an intensity of about 1.5
W/cm² (at a frequency of 1 MHz) has already been powered by a rechargeable battery (Henley
International, Sonopuls 434), one could envision the possibility of designing battery operated
portable sonicators. With further research (see the next chapter), one might envision small
pocket-size sonicators carried by the patients and used to "inject" drugs whenever required.
8. Conclusions and Future Directions

8.1. Conclusions: Application of therapeutic ultrasound (1-3 MHz, 0-2 W/cm²) has been used to enhance transdermal transport of more than 15 drugs over the last 40 years. However, the observed enhancement of transdermal transport varies from drug to drug. In many cases, no enhancement was measured. This raised a controversy in the literature regarding the applicability of phonophoresis as a transdermal transport enhancer. This uncertainty and inefficiency of phonophoresis (local delivery) and sonophoresis (systemic delivery) motivated the mechanistic studies reported in this thesis.

Various phenomena, including cavitation, thermal effects, generation of convective velocities, and mechanical effects, were analyzed as possible mechanisms of sonophoresis. Cavitation-induced lipid bilayer disordering was found to be the most important cause for ultrasonic enhancement of transdermal transport. The observed enhancement depends significantly on the chemical nature of the permeant. A theoretical model was developed to predict sonophoretic transdermal transport enhancement. The theoretical predictions, as well as the measured sonophoretic enhancements of different permeants, suggest that the permeants passively diffusing through the skin at a slow rate are most enhanced by the application of ultrasound. These findings provide quantitative guidelines for estimating the efficacy of sonophoresis in enhancing transdermal drug delivery. It was shown, using the model, that the typical enhancement induced by therapeutic ultrasound is about 10-fold. This limitation arises due to the small fraction of bilayers disordered by ultrasound application. In addition, the transport in the presence of therapeutic ultrasound occurs through lipid bilayers (partially disordered by ultrasound application). Accordingly, transdermal transport is proportional to the lipophilicity of drugs and prohibits transdermal transport of hydrophilic drugs.

The conclusion that cavitation plays a major role in sonophoresis suggested that low-frequency sonophoresis should be more effective than therapeutic ultrasound. Indeed, low-frequency ultrasound enhanced transdermal transport of a variety of drugs across human cadaver skin in vitro by a factor of up to 5000. In vivo experiments performed using salicylic acid as a model drug show that low-frequency ultrasound enhances transdermal transport across living skin by a factor of at least 300. Application of low-frequency ultrasound does not appear to cause any long-term damage to the barrier properties of the skin as well. In addition, preliminary histological studies indicate no damage to the skin and underlying tissues of
hairless rats exposed to ultrasound, although further studies are required before arriving at any final conclusions regarding the safety of low-frequency ultrasound. It was found that the sonophoretic permeability of several compounds examined was remarkably similar in spite of the fact that their partition coefficients differed by up to 10000-fold. This behavior is different from that exhibited by the passive skin permeability which decreases dramatically with increasing permeant molecular weight and decreasing lipophilicity, thus allowing only few drugs to permeate the skin at a therapeutically significant rate. The observed independence of the sonophoretic skin permeability on permeant characteristics suggests that low-frequency sonophoresis may potentially provide a method for the delivery of a wide variety of compounds irrespective of their physico-chemical properties. A mechanistic explanation for low-frequency sonophoresis was proposed. It was suggested that application of low-frequency ultrasound generates aqueous channels across the SC, through which transdermal transport of drugs may occur. This hypothesis explains the observation that the sonophoretic permeability of various drugs is strikingly similar in spite of the fact that their physico-chemical characteristics are markedly different. A quantitative model was developed to predict the effect of low-frequency ultrasound on transdermal transport of drugs. The predictions of the model are reasonable quantitative agreement with the experimental observations.

Low-frequency ultrasound was also found to increase the permeability of human skin to high-molecular weight proteins, thus making transdermal administration of these molecules potentially feasible. Specifically, therapeutic doses of proteins such as insulin, \( \beta \)-interferon, and erythropoietin could be delivered across human skin in vitro and insulin across hairless rat skin in vivo. This makes low-frequency ultrasound a potential non-invasive substitute for traditional methods, such as injections, although significant further research is required in order to achieve this objective. This is discussed next.

**8.2. Future Research Directions:** Further fundamental research should be performed to address two issues: i) visualization of the aqueous channels generated in the lipid bilayers of the SC during low-frequency sonophoresis. These studies could be performed using microscopic techniques such as Scanning Electron Microscopy or Fluorescence microscopy, and ii) examination of the variation of the sonophoretic skin permeability with the permeant molecular weight. These studies will advance our understanding of the limiting protein size that sonophoresis can deliver transdermally, as well as assist in the assessment of whether sonophoresis may deliver viruses across the skin, which is an important issue from a safety point of view. The investigations of the size-dependence of sonophoretic skin permeability could be performed by experimentally measuring the sonophoretic permeabilities to several
proteins possessing molecular weights in the range of 50,000 to 1000,000 Da, as well as to viruses.

Research also needs to be pursued in four main areas in view of the practical applications of low-frequency sonophoresis. These include: i) optimization studies, ii) safety studies, iii) investigations of patient compliance related issues, and iv) clinical trials. Specific issues in each category and some initial thoughts on how to address these issues are discussed next.

i) **Optimization of Low-Frequency Ultrasound Parameters**: Using the ultrasound conditions discussed in this thesis (20 KHz, 225 mW/cm², 10% duty cycle) the required transdermal patch area to deliver therapeutic doses of proteins is about 40 cm² and the estimated required time is about 1 hour. Attempts should be focused on optimizing ultrasound parameters including frequency, intensity, and duty cycle to minimize the patch area as well as the application time. The optimization studies should focus on in vitro as well as in vivo studies of the efficacy of ultrasound in enhancing protein, for example, insulin, transport under different combinations of ultrasound conditions. Safety studies should also be performed under each combination of ultrasound parameters to ensure a safe ultrasound exposure.

In addition to the optimization of ultrasound parameters, the possibility of using low-frequency ultrasound along with other enhancers, for example, electricity, should also be explored. As discussed earlier, application of low-frequency ultrasound induces formation of aqueous channels across the SC. It is conceivable that a simultaneous application of small electric currents may induce electrophoretic transport through these channels, thus enhancing the effect of ultrasound on transdermal transport.

ii) **Safety**: Further assessment of the effects of single as well as multiple ultrasound exposures on the skin should be undertaken. The latter is of special importance for the delivery of insulin, since the patients may be exposed to ultrasound chronically. Experiments should also be performed to assess the effect of ultrasound on the stability of proteins that will be delivered transdermally. Attention should be focused on the possible degradation of proteins due to shearing or radical formation induced by ultrasound. To estimate possible protein degradation, protein solutions at a known concentration could be placed in a beaker and could be exposed to ultrasound under a variety of conditions. The activity of proteins in solution should be assayed periodically using ELISA or RIA, HPLC, or biological activity (for example, decrease in the blood glucose level of hairless rats upon insulin administration). In addition, the possibility of inducing immunogenicity of transdermally delivered proteins using ultrasound should also be explored.
iii) **Patient Compliance Issues:** This includes investigations of the possible pain induced by low-frequency ultrasound. The examination of possible pain induced by low-frequency ultrasound could be performed in animal models using the following protocol. Hairless rats could be first trained not to respond (as judged by flexural withdrawal) to an ultrasound probe (turned OFF) on their lower extremity. Then one could examine whether the rats respond to the ultrasound probe after turning it ON. The response could be judged by the time taken by the hairless rat for withdrawal from the probe. No difference in the withdrawal time upon turning ultrasound ON would suggest no pain, while a difference would suggest that some pain is associated with ultrasound application.

Patient compliance related studies should also include the examination of the possibility of manufacturing portable sonicators. Note that a knowledge of the optimal conditions to be used for sonophoresis is critical in order to investigate the possibility of designing a small device. Once the optimal conditions are known, a piezo-electric crystal having a resonating frequency corresponding to the optimal frequency could be purchased from either Crystal Biotech or Sonics and Materials. Based on knowledge of the required optimal ultrasound intensity for sonophoresis, the required ultrasound output power can be calculated by multiplying ultrasound intensity by the required transducer area which is equal to the required transdermal patch area. The electrical power required to achieve the given ultrasound power can be calculated by dividing the required ultrasound power by the efficiency of the piezo-electric crystal. Once the required power output from the signal generator is known, pocket-size signal generators and amplifiers delivering the required power can be manufactured or purchased with technology similar to that used in manufacturing a radio or a walkman.

iv) **Clinical trials:** Clinical trials should be performed to assess the efficacy of low-frequency ultrasound in enhancing protein transport across human skin in vivo. Protocols for initial clinical studies of transdermal insulin delivery have been submitted to the Massachusetts General Hospital and have been approved by the Institutional Review Board.
9. Bibliography


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