Hearing Loss induced by Bacterial Meningitis

Investigations into the possible involvement of :

i) Bacterial Ototoxins

ii)Nitric Oxide, Excitotoxicity, and Reactive Oxygen Species

by

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ABSTRACT

Background

At the commencement of this work there was evidence to suggest that a bacterial ototoxin, pneumolysin, was at least partly responsible for meningitis-induced hearing losses caused by *Streptococcus pneumoniae* type II; preliminary evidence existed for an ototoxin produced by *Haemophilus influenzae type b* (Hib).

There was also evidence to suggest that:

(i) excitatory amino acids (EAAs), nitric oxide (NO) and reactive oxygen species (ROSs) may also be involved in hearing losses induced by meningitis;

(ii) N-methyl-D-aspartic acid (NMDA) and non-NMDA receptors are involved in excitatory damage produced by EAAs in the cochlea, with non-NMDA receptors playing a predominant role;

(iii) NO and ROSs mediated the ototoxic effects which ensue from activation of NMDA receptors in the cochlea.

There were no reports concerning the possible involvement of NO and ROSs in the ototoxic effects which ensue from activation of non-NMDA receptors.

Summary of this work

All bacterial extracts and other chemicals were perfused into the cochlea of anaesthetised guinea pigs and the level of ototoxicity of each extract/chemical was determined by recording auditory evoked potentials.

1. Attempts were made to demonstrate/identify ototoxins in cytoplasmic contents of *S. pneumoniae* type III, Hib *and Neisseria meningitidis.*

Ototoxic activity was demonstrated in extracts of Hib, *N. meningitidis* and wild type *S. pneumoniae* type III. Tentative evidence was adduced to demonstrate the presence of a 'pneumolysin-like' protein in extracts of Hib and *N. meningitidis*. The amounts of this protein

present, estimated by inspection of SDS gel analyses of comparable gel loadings, reflected the ototoxic potency of these meningitogenic bacteria: *S. pneumoniae* > Hib> *N. meningitidis* (trace). In addition, extracts from a pneumolysin-deficient mutant of *S. pneumoniae* type III were ototoxic as were preparations of wild type extracts immunopurified with anti-pneumolysin immunosorbents; this suggests the possibility of another ototoxin in *S. pneumoniae*.

2. Evidence points to the possibility that excitotoxicity in the cochlea during meningitis contributes to some (particularly transient) hearing losses probably involving activation of non-NMDA, as well as NMDA receptors. If the non-NMDA receptors were definitively shown to be involved in the induction of deafness, it is unlikely that such effects would be mediated by NO, and ROSs, for the following reasons. NO synthase inhibitors (L-nitro arginine methyl ester (L-NAME) and L-methyl arginine (L-MA)) and ROS scavengers (Superoxide dismutase (SOD) and deferoxamine (DEF)) afforded no protection on cochleae from the excitotoxic actions of kainate and quisqualate - known agonists of non-NMDA receptors - as judged by electrophysiological and morphological criteria.

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Abbrieviations

ABERs	Auditory Brainstem Evoked Responses
Ach	Acetylcholine
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Proprionic Acid
APL	Artificial Perilymph
ATP	Adenosine Trisphosphate
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CAP	Compound Action Potential
cGMP	Cyclic Guanosine Monophosphate
CM	Cochlear Microphonic
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DEAE	Diethylaminoethyl
DEF	Deferoxamine
DTT	Dithriothreitol
EAA	Excitatory Amino Acid
EDTA	Ethylenediaminetetraacetic Acid
GABA	γ-Aminobutyrate
Hib	*Haemophilus influenzae* Type b
Ig	Immunoglobulin

IL-1	Interleukin 1
Kainate	Kainic Acid
L-Ma	Methyl-L-Arginine
L-Name	Nitro-L-Arginine Methyl Ester
LOS	Lipo-Oligosaccharide
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
Meningococcus	*Neisseria meningitidis*
NMDA	N-Methyl-D-Aspartic Acid
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OAEs	Otoacoustic Emissions
PAF	Platelet Activating Factor
PAGE	Polyacrylamide Gel Electrophoresis
PMNs	Polymorphonucleocytes
Pneumococcus	*Streptococcus pneumoniae*
PRP	Polyribosylribitolphosphate
ROSs	Reactive Oxygen Species
SAS	Sub Arachnoid Space
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SNP	Sodium Nitroprusside
SOD	Superoxide Dismutase

SPL	Sound Pressure Level
TA	Teichoic Acid
TEM	Transmission Electron Microscopy
TNF	Tumour Necrosis Factor
WBC	White Blood Cell

CHAPTER 1

Introduction

1.1. Meningitis

Meningitis is an infection of the meninges which can be caused by both viral and bacterial pathogens. There are approximately 3000 reported cases of bacterial meningitis each year in the UK (National meningitis trust 1992). Despite the development of antibiotics, the rapid time course of the infection and poor immunity within the cerebrospinal fluid (CSF) leads to a high incidence of mortality and neurological sequelae in survivors. Hearing loss is one of the most common sequelae of bacterial meningitis.

The initial symptoms of bacterial meningitis may progress over one or two days, but can develop very rapidly, sometimes in a matter of hours. Therefore prompt diagnosis and antibiotic treatment is essential in order to facilitate a full recovery. Initial diagnosis of bacterial meningitis is often difficult because there are a number of different symptoms, and many of these are also symptoms of flu. They include headache, fever, vomiting, neck stiffness, drowsiness, dislike of bright lights and a purpuric rash. In addition, babies may have high pitched/moaning cry, pale or blotchy skin, refuse feeds, and can be fretful or difficult to wake. If seizures or coma develop the prognosis is poor. Clinically, the diagnosis is confirmed by sampling of the CSF. Bacteria can be identified by CSF culture or antigen detection. Classically, the CSF is turbid due to leukocytosis (white blood cells in the CSF), the CSF protein concentration is high, and the ratio of CSF/plasma glucose concentration is low. Since CSF abnormalities are not always apparent, definitive diagnosis rests with identification of bacteria in the CSF. Upon diagnosis, patients should receive anti-microbial therapy immediately which is commonly a combination of penicillin and chloramphenicol. In the case of multi-resistant bacterial strains, third generation cephalosporins are the frequent choice. Chemoprophylaxis is recommended for close contacts in order to eliminate carriers, and Rifampicin is usually used.

There are three main pathogens which cause bacterial meningitis: *Streptococcus pneumoniae* (pneumococcus), *Haemophilus influenzae* type b (Hib), and *Neisseria meningitidis* (meningococcus). **Pneumococci** are gram positive dipplcocci (1μm diameter) which are often arranged in chains. They are facultative anaerobes and are readily lysed by surface active agents such as bile salts. There are at least 84 capsular types (Brooks, Butel and Ornston 1995) serotyped according to immunologically distinct capsular polysaccharides. **Haemophilus influenzae** are gram negative short pleomorphic coccobacilli (1x1.5x0.3 μm) which are fastidious facultative anaerobes (Brooks *et al.* 1995). There are 6 types of capsulated *Haemophilus influenzae* subtyped a-f. The six subtypes can be serologically typed according to capsular polysaccharides with specific antiserum by agglutination or Quellung reaction. *H. influenzae* type b is responsible for 95% invasive diseases caused by *H. influenzae*. **Meningococci** are gram negative oval dipplococci (0.8μm diameter) and are exacting in their growth requirements (Brooks *et al.* 1995); some are facultative anaerobes. There are more than 8 serotypes of meningococci which can be serotyped according to their polysaccharide capsular antigens. The most important serotypes in causing disease are types A, B, C, Y and W135. Group B *N. meningitidis* is currently the prevalent meningeal pathogen within the UK and is associated with both local outbreaks of the disease as well as epidemics.

S. pneumoniae, *H. influenzae* and *N. meningitidis* were responsible for 13, 48 and 20 % of reported bacterial meningitis cases in the United States between 1978 and 1981 (Schlech, Ward, Band *et al.* 1985). Of the remaining (19%) cases, 6% were caused by unknown pathogens. Neonatal bacterial meningitis was most commonly evoked by *Streptococcus agalactia*, and *S. pneumoniae* was the main pathogen responsible for meningitis in patients more than 60 years old. *H. influenzae*, *N. meningitidis* and *S. pneumoniae* were responsible for the majority of cases between these ages (Schlech *et al.* 1985). These data demonstrate that the predominant etiologic agent of bacterial meningitis varies with the age of the host. The incidence of *H. influenzae* type b meningitis is now falling dramatically due to the success of widespread vaccination with conjugate *H. influenzae* type b vaccines (Greenwood, Slack and Peutherer 1997), described later in this Chapter. Therefore *S. pneumoniae* and *N. meningitidis* are likely to be the leading cause of bacterial meningitis in future reports. This is supported by a recent report of 124 bacterial

meningitis cases in children aged between 4 weeks and 16 years in the UK. Only 1 case was caused by *H. influenzae* type b whereas *N. meningitidis* and *S. pneumoniae* were responsible for 92 and 18 cases respectively (Richardson, Reid, Tarlow *et al.* 1997). This is in direct contrast to the pre-*H. influenzae* type b vaccine reports in which *H. influenzae* type b was the predominant causative agent of bacterial meningitis in children of this age (Schlech *et al.* 1985).

1.1.1. Experimental Study of the Pathogenesis of Bacterial Meningitis

Several animal models have been developed in order to study the pathological events that occur during bacterial meningitis. The two most common models are the *infant rat* and the *adult rabbit* models of meningitis (Tauber and Zwahlen 1994). The **infant rat model** of meningitis most closely resembles meningitis in humans and is of particular use for studying the early pathological events (reviewed in Tauber and Zwahlen 1994). In this model, bacterial meningitis frequently develops following intranasal inoculation of bacteria, and the whole course of the infection from the initial nasopharyngeal colonisation can be studied. Unfortunately, unless primates are used, frequent sampling of the CSF is not possible in infant animals, and larger adult animals rarely develop meningitis following intranasal inoculation. Therefore an **adult model of meningitis** has been developed in which bacteria are inoculated directly into the blood or CSF. This bypasses the initial stages of colonisation and invasion into the blood stream which can be age dependent, and allows the frequent sampling of CSF. Although this is an artificial route of infection, it produces a consistent infection with a reproducible time course (Bhatt, Halpin, Hsu *et al.* 1991). In addition, meningitis can be induced by bacterial products and inflammatory mediators allowing investigation of the role of specific individual factors in the pathogenesis of bacterial meningitis (Tuomanen, Lui, Hengstler *et al.* 1985). The pathogenicity of *H. influenzae* has been most commonly studied in the infant rat model of meningitis (Moxon, Smith, Averill *et al.* 1974; Kaplan, Hawkins, Kline *et al.* 1989; Wiederman, Hawkins, Johnson *et al.* 1986), but has been also studied in mice (Schneerson, Rodriguez, Parke *et al.* 1971), rabbits (Osborne, Comis, Tarlow *et al.* 1995: Tuomanen, Saukkonen, Sande *et al.* 1989) and primates (Daum, Sheifele, Syriopoulou *et al.* 1978). Experimental pneumococcal meningitis has been induced in rats

(Strake, Mitten, Ewing *et al.* 1996), guinea pigs (Winter, Comis, Osborne *et al.* 1997), dogs (Scheld, Park, Dacey *et al.* 1979) and cats (Hochwald, Nakamura, Chase *et al.* 1984), but has been most commonly induced in adult rabbits (Bhatt *et al.* 1991; Tuomanen *et al.* 1989). Experimental meningococcal meningitis has been induced in adult rabbits (Tuomanen *et al.* 1989), infant rats (Saukkonen 1988), and most commonly in mice (Salit and Tomalty 1986). Animal models of meningococcal infections have proved difficult because of the relative avirulence of this pathogen in hosts other than humans. Mice have been the most common choice because there are well characterised lines of inbred mice which are immunologically and genetically susceptible to meningococcal infection. In addition, gastric mucin has been used to enhance infections by providing more available iron for bacterial growth (Arko 1989).

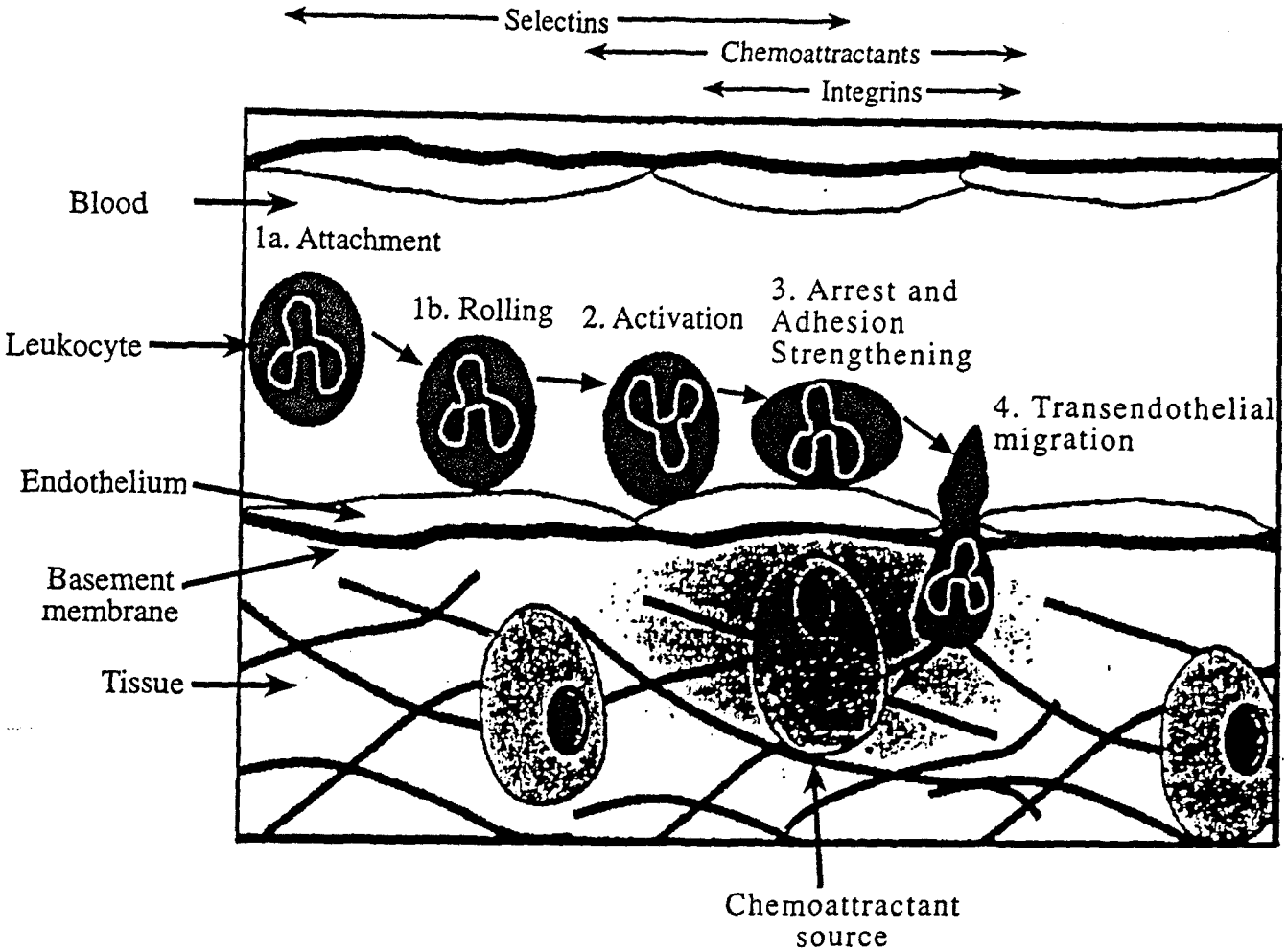
In vitro studies using cultured human cells, and organ cultures have also been used to study the pathogenesis of Hib, pneumococcus and meningococcus (Ring, Weiser and Tuomanen 1998; Stephens 1989; Stephens and Farley 1991; St Geme and Cutter 1995).

1.1.2. Pathogenesis of Bacterial Meningitis

Bacteria enter the body at the nasopharynx. There is an **initial colonisation of the nasopharynx** which most commonly results in an asymptomatic carrier state, but occasionally results in invasion into the submucosa and bloodstream. Use of isogenic mutants of *H. influenzae* type b demonstrate that organisms which gain access to the blood and central nervous system (CNS) are the progeny of a small fraction of the original challenge inoculum, probably a single bacterium (Moxon and Murphy 1978). Virulent bacteria have evolved divergent mechanisms for colonisation and invasion, some of which are common to *S. pneumoniae*, *H. influenzae* type b, and *N. meningitidis*. These include, attachment to mucus and to nonciliated epithelial cells, ciliostasis, cytotoxicity, multiplication, and invasion of the nasopharyngeal mucosal surfaces. **Invasion of the nasopharynx** by *H. influenzae* type b is intercellular involving breakdown of epithelial cell tight junctions between epithelial cells, whereas meningococci invade the nasopharynx by endocytic internalisation (Stephens and Farley 1991). The mechanism by which pneumococci gain access to the blood stream is not clear (Boulnois 1992).

Once meningitogenic bacteria reach the blood stream, the course of the disease depends on the ability of host defences to clear the bacteria. If host defences are insufficient bacteria multiply and then proceed to invade the CNS. Since other bacterial species produce continuous bacteraemia without inducing meningitis, there must be virulence factors involved in invasion of the meninges which are unique to meningitogenic bacteria. Current evidence suggests that bacteria enter the CSF in the lateral ventricles, presumably through the choroid plexi (Daum *et al.* 1978). The mechanism by which bacteria gain access to the CSF is not clear, but several theories have been put forward. The Trojan horse theory suggests that bacteria gain access to the CSF in association with monocytes migrating along their normal pathways. Transmission electron microscopic studies indicate that *H. influenzae* type b cross the endothelial barrier by direct invasion of the endothelial cells similar to that reported for endothelial cells of the human umbilical vein (Virji, Kayhty, Ferguson *et al.* 1991). Recent studies have shown that pneumococcal invasion of rat and human brain microvascular endothelial cells is intracellular and is likely to be mediated by the interaction of platelet activating factor (PAF) receptors in the expressed by the endothelial cells with choline binding proteins in the pneumococcal cell wall (Ring *et al.* 1998).

Since host defences within the CSF are inadequate, bacteria in the CSF are able to multiply rapidly. **Leukocytosis** follows bacterial invasion of the CSF. Current opinion is that leukocytes invade the CSF by a 3 step process (Springer 1994). Initially leukocytes roll along the endothelial cells of the blood-brain barrier (BBB), they then adhere firmly to the endothelial cells and later traverse the BBB (transmigration). The first step is achieved by the presence of selectins on the endothelial lining of the BBB and counter carbohydrate receptors on the leukocytes. The second and third steps are achieved by i) the interaction of chemoattractors (displayed or released on the endothelium of blood vessels) with specific leukocyte membrane receptors, and ii) binding of integrins (on leukocyte membranes) with immunoglobulin (Ig) superfamily members on the endothelium (Springer 1994) (**Figure 1.1.2.**). Selectins are dramatically mobilised or synthesised in response to cytokines, and leukocytes are only adhesive to the BBB when activated by external stimuli. Therefore it is thought that bacterial invasion into the CSF causes a local release of inflammatory mediators which stimulates expression/mobilisation of selectins, and



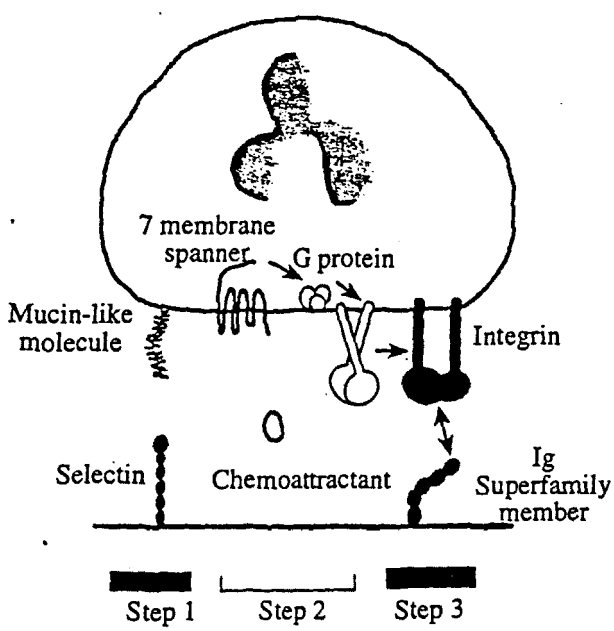


Figure 1.1.2. Diagrams to illustrate the 3 step process of leukocytosis.

1. Selectin molecules that bind on carbohydrate ligands, often displayed on mucin-like molecules, are responsible for the inital tethering of a flowing leukocyte to the vessel wall and for labile, rolling adhesions. Tethering brings leukocytes into proximity with chemoattractants that are displayed on or released from the endothelial lining of the vessel wall.

2.Chemoattractants bind to receptors that span the membrane seven times on the surface of leukocytes. These couple to G proteins, which transduce signals that activate integrin adhesiveness. The integrins can then bind to Ig superfamily members on the endothelium, increasing adhesiveness and resulting in arrest of the rolllng leukocyte.

3. Following directional cues from chemoattractants and using integrins for traction, leukocytes then cross the endothelial lining of the blood vessel and enter the tissue.

(Taken from Springer 1994)

display/release of chemoattractors. These factors enable leukocytes with the appropriate counter receptors and integrins to extravasate into the sub arachnoid space (SAS).

A **break down in the BBB** also follows bacterial invasion into the CSF and is characterised by an accumulation of proteins within the CSF. The BBB is located at the cerebral microvascular endothelial cells and exhibits functional and morphological differences to other endothelial cells. Cerebral endothelial cells possess narrow intercellular tight junctions which hinder paracellular transport of hydrophilic compounds, and pinocytotic vesicular transport is virtually absent which implies that fluid phase uptake is limited (De Vries, Kuiper, De Boer *et al.* 1997). During experimental *H. influenzae* type b meningitis in rats a separation of the tight junctions and an increase in the number of pinocytotic vesicles, correlated with penetration of albumin into the CSF has been observed (Quagliarello, Long and Scheld 1986). The mechanism by which this damage to the BBB occurs is not clear.

Bacterial invasion of the CSF results in **cerebral oedema** of multiple origin which is responsible for the **increase in intracranial pressure** associated with meningeal infections. *Vasogenic oedema* results from increased leakage of fluid from cerebral capillaries due to breakdown of the BBB. *Cytotoxic oedema* results from swelling of cellular elements due to electrolyte changes caused by leukocytes and/or bacterial toxic factors. Increased CSF levels of antidiuretic hormone can also contribute to cytotoxic oedema by diluting the extracellular fluid and increasing the permeability of the brain to water. In addition *interstitial oedema* results from obstruction of the drainage of CSF into the blood due to dysfunction of arachnoid villi by entrapment of leukocytes.

Secondary bacteraemia may result from local CNS suppurative processes allowing bacteria to continuously enter and leave the CSF. This is often seen in experimental meningitis in which bacteraemia is produced following intracisternal inoculation (Winter 1997). When the blood vessels of the brain become infected with bacteria (**vasculitis**), narrowing of the vessels or thrombus formation can occur, leading to ischaemia or infarction of the brain. Vasospasm can also occur via humoral factors. If large arteries in the base of the brain are involved **severe neurological complications** can arise (Igrashi, Gilmartin, Gerald *et al.* 1984). Impeded blood

flow to the brain results in an increased concentration of lactate and decreased concentration of glucose in the blood which is important in diagnosis of clinical bacterial meningitis.

Eventually, one or more of the physiological alterations evoked by meningeal invasion results in neuronal injury and irreversible focal or diffuse brain damage.

1.1.3. Cytokines and Inflammation

During meningitis there is a compartmentalised local release of **inflammatory mediators** within the CSF in response to bacteria and bacterial products. The ensuing response is produced by a complex cascade involving hosts cells and many inflammatory mediators including polymorphonucleocytes (PMNs), proinflammatory mediators (cytokines and PAF), excitatory amino acids (EAAs) and reactive oxygen species (ROSs).

The two main cytokines which initiate the inflammatory response are *Interleukin 1* (IL-1) and *Tumour Necrosis Factor* α (TNFα) (reviewed in Saez-Llorenz, Ramillo, Mustafa *et al.* 1990). These are found in the CSF of most patients with bacterial meningitis, but not in the CSF of control or viral meningitis patients (Mustafa, Lebel, Ramillo *et al.* 1989a). **IL-1** is produced and released by mononuclear phagocytes activated by bacterial products such as cell wall components and toxins (Riesenfeld-Orn, Wolpe, Garcia-Bustos *et al.* 1989). Other cells including microglia, astrocytes and cerebral endothelial cells are also capable of producing IL-1 (De Vries *et al.* 1997). The presence of IL-1 in the CSF correlates with presence of TNFα, and adverse outcome. High levels of IL-1 have been correlated with neurological sequelae (Mustafa *et al.* 1989a). Intracisternal injection of IL-1 alone is capable of inducing inflammation similar to that seen in bacterial meningitis including leukocytosis and an increase in permeability of the BBB (Quagliarello, Wispelway, Long *et al.* 1991). **TNFα** is produced and released primarily by monocytes and macrophages, although other cells including astrocytes, microglial cells and cerebral endothelial cells are capable of TNFα production (De Vries *et al.* 1997). It is produced in response to multiple stimuli including microbial or immunological insults, particularly endotoxin (Mustafa, Ramilo, Olsen *et al.* 1989b). In physiological concentrations, TNFα is believed to

benefit the host by aiding the hosts immune system and assisting tissue repair. However, at greater concentrations, TNFα can lead to fatal toxic reactions similar to that seen by administration of endotoxin or infection with gram negative bacteria. Intracisternal injection of TNFα induces leukocytosis and alterations in the BBB (Kim, Wass, Cross *et al.* 1992), and in combination with IL-1 is synergisitic in inducing meningeal inflammation (Quagliarello *et al.* 1991). In experimental models of meningitis, administration of anti-TNFα monoclonal antibody attenuates the inflammatory response to Hib endotoxin (Mustafa *et al.* 1989b).

Phospholipase A2 is responsible for the production of two main inflammatory pathways: one which results in the production of **PAF** and a second (the arachidonic acid pathway) which results in the production of **prostaglandins, thromboxanes** and **leukotrienes (eicosanoids)**. In outline, leukotrienes cause PMN chemotaxis which results in leukocytosis, and, PAF and thromboxanes promote thrombosis in the microcirculation which ultimately results in regional cell death. Phospholipase A2 is activated on membrane phospholipids of many cells including neutrophils, platelets and vascular endothelial cells in response to bacterial (and other) antigens. Since IL-1 and TNFα appear to induce phospholipase A2 activity, some of the effects of these cytokines are likely to be mediated by the production of PAF and eicosanoids (Saez-Llorenz *et al.* 1990).

Polymorphonucleocytes enter the CSF during meningitis in response to cytokines. Although PMNs are essential in eradicating bacteria from the blood, experimental studies indicate that the action of PMNs in the CSF is more detrimental than beneficial. This is clearly seen in experimental meningitis in rabbits induced by live *S. pneumoniae, N. meningitidis, H. influenzae*, pneumococcal cell wall or lipopolysaccharide (LPS) in which inhibition of leukocytosis by administration of monoclonal antibodies to the CS18/11 adhesion promoting receptors significantly reduced the alteration of permeability of the BBB, delayed secondary bacteraemia, and prevented mortality and cerebral oedema (Tuomanen *et al.* 1989). Since PMNs rely on antibodies and cytokines to select targets for their action, the lack of opsonins and complement in the CSF means that the action of PMNs is less well targeted and results in damage of host tissues. Toxic agents released by PMNs include ROSs, nitric oxide (NO), proteases and glutamate.

There is now accumulating evidence that **nitric oxide** or a secondary oxidant derived from nitric oxide is involved in the pathophysiological events during meningitis. Several clinical studies have reported increased levels in CSF of nitrite and nitrate (products derived from NO) in patients with bacterial meningitis (Kornelisse, Hoekman, Visser *et al.* 1996a; Van Furth, Seijmmonbergen, Groeneveld *et al.* 1996). Moreover, Kornelisse *et al.* (1996a) reported that elevated nitrite and nitrate levels in CSF correlate with TNFα and glucose levels, and a decrease in the level of l-arginine (precursor of NO). Increased levels of nitrates in the CSF have also been reported during experimental meningitis and were correlated with increased permeability of the BBB (Buster, Weintrob, Townsend *et al.* 1995).

Several studies have been carried out using various nitric oxide synthase (NOS) inhibitors to determine whether NO is involved in the inflammatory response during experimental meningitis (reviewed in Pfister and Scheld 1997): NOS inhibitors attenuate the inflammatory response in the early phase of meningitis (Buster *et al.* 1995; Boje 1995; Boje 1996; Koedel, Bernatowicz, Paul *et al.* 1995). However, the effects of such inhibitors on the more advanced stages of meningitis is less clear. For example, NOS inhibitors considerably worsened neuronal injury *in vivo* (Leib, Kim, Black *et al.* 1996), whereas NOS inhibitors attenuated toxicity in astrocytes and microglia produced by gram positive cell walls *in vitro* (Kim and Tauber 1996).

The source of NO production during meningitis is not known. Bacterial production of NO/nitrate is unlikely since nitrite and nitrate levels are raised in meningococcal meningitis patients and meningococci do not form NO (Vanfurth, Despland, Lutschg *et al.* 1996), and, increased nitrite levels are seen in experimental models of meningitis using endotoxin alone (Buster *et al.* 1995; Korytko and Boje 1996) or heat killed pneumococci (Koedel *et al.* 1995). A number of host cells are capable of producing NO by NOS, including endothelial cells, astrocytes, microglia, cerebellar neurones, and white blood cells (Korytko and Boje 1996). There are two types of constitutively produced NOS, endothelial (type III) and neuronal (type I) NOS, which produce small amounts of NO in response to an increase in intracellular Ca^{2+}. A third inducible NOS (type II NOS) is expressed in response to a specific combination of cytokines (including IL-1 and

TNFα) by a number of cells including smooth muscle cells, macrophages and glial cells. Inducible NOS (iNOS) synthesises large amounts of nitric oxide until it is inactivated by another specific combination of cytokines (including interleukins 4, 8, and 10, TNFβ, and platelet derived factors) (Mayer and Hemmens 1997). Therefore inducible NOS would seem to be a likely candidate as a tissue-damaging determinant in meningitis. The time course and effect of NOS inhibitors on NO production during experimental meningitis supports the concept that iNOS is responsible (Korytko and Boje 1996; Boje 1996). However, dexamethasone is an inhibitor of inducible NOS, and its administration was not associated with reduced levels of nitrite in the CSF of patients with bacterial meningitis (Kornelisse *et al.* 1996a). Therefore neuronal and endothelial NOS may also be involved in the increased production of NO during meningitis.

In conclusion, NO appears to play an important role in the pathogenesis of early events associated with bacterial meningitis However, further investigations are necessary in order to elucidate the exact source and site of action of NO produced during bacterial meningitis. Since previous studies have used non-selective NOS inhibitors, more selective inhibitors of nitric oxide should help to clarify the situation.

Reactive oxygen species (ROSs) are by-products of aerobic metabolism which have the potential to disrupt cellular function and structure. A number of different anti-oxidants have been shown to produce beneficial effects on the inflammation associated with bacterial meningitis (reviewed in Pfister and Scheld 1997). Scavengers of hydrogen peroxide, superoxide, and hydroxyl radicals have all been shown to attenuate the early inflammatory response in experimental meningitis. For example, Koedel *et al.* (1995) demonstrated that superoxide dismutase (scavenger of superoxide) produced attenuation of brain water content, intracranial pressure and CSF white blood cell count similar to that seen by inhibition of NOS. Therefore ROSs appear to play an important role in the early pathogenic events of bacterial meningitis.

CSF concentrations of **excitatory amino acids** are elevated in human clinical and in rabbit experimental bacterial meningitis (Guerra-Romero, Tureen, Fournier *et al.* 1993), and are

correlated with the severity of the disease (Spranger, Schwab, Krempien *et al.* 1996). Moreover, in experimental meningitis models, a non-specific glutamate receptor antagonist attenuated neuronal injury (Leib, Kim, Ferriero *et al.* 1996), and a non-competitive NMDA receptor antagonist attenuated oedema and BBB injury (Bass, Engelhard, Trembovler *et al.* 1996). In addition, toxicity of cerebellar neurones produced by macrophages *in vitro* was blocked by NMDA receptor antagonists, but not by catalase or SOD (Piani, Spranger, Frei *et al.* 1992). Therefore EAAs also appear to play a role in the pathogenesis of bacterial meningitis. The source of excitatory amino acids during meningitis is unclear. Since re-uptake of amino acids is energy dependent at the synapse, ischaemia could lead to accumulation of the amino acids. In addition, increased permeability of the BBB to excitatory amino acids, release of amino acids from PMNs or cells in the CNS, decreased clearance of amino acids from the CSF, and increased binding of amino acids to brain tissue could all contribute to the deleterious effects seen in meningitis (Pfister, Fontana, Tauber *et al.* 1994).

In conclusion, many different mediators are involved in the inflammatory response produced during bacterial meningitis. The precise interaction of each of these factors is not clear. Despite some bacterial killing by the inflammatory response, clinical and experimental data clearly indicate that the majority of the deleterious consequences of bacterial meningitis are a direct consequence of this inflammatory response by the host (Townsend and Scheld 1996). Antibiotics ultimately reduce inflammation by eliminating the bacteria, however, initially they can worsen the inflammation by releasing bacterial products which stimulate the release of inflammatory mediators. For example, the fraction of free endotoxin in CSF increases 10-fold during bacterial death induced by antibiotics which act on the bacterial cell wall (Arditi, Ables and Yogev 1989; Tauber, Shibl, Hackbarth *et al.* 1987). It is during this critical stage at which antibiotics are administered that control of the inflammatory response would be most beneficial.

1.1.4. Control of the Inflammatory response

There is now evidence of an increase in naturally occurring anti-inflammatory mediators during meningitis. **Interleukin 10** (IL-10) is an endogenous interleukin which suppresses the synthesis of pro-inflammatory cytokines. It is produced by monocytes/macrophages, and lymphocytes and

its concentration in the CSF and CSF/blood ratio is elevated in children with bacterial meningitis (Kornelisse, Savelkoul, Mulder *et al.* 1996). Systemically administered IL-10 attenuated the increase in regional cerebral blood flow, brain water content, intracranial pressure and CSF white blood cell count in experimental pneumococcal meningitis (Koedel, Bernatowicz, Frei *et al.* 1996). In addition, systemic and intrathecal administration of IL-10 reduced the TNFα and lactate levels in CSF in experimental meningitis induced by *H. influenzae* type b or *Listeria monocytogenes* (Paris, Hickey, Trujillo *et al.* 1997). There are also naturally occurring **TNFα antagonists**. These are soluble extracellular domains of the 55 and 75 kDa membrane bound TNF receptors (sTNFR-55 and sTNFR-75) which neutralise the activity of TNFα. **sTNFR-55** and **sTNFR-75** are shed from the surface of PMN cells and monocytes in response to many of the same inflammatory stimuli which are known to induce TNFα release, and IL-10 stimulates their release from monocytes *in vitro*. In clinical meningitis the levels of sTNFR-55 and sTNFR-75 are significantly raised (Kornelisse *et al.* 1996b). Therefore it is likely that both IL-10 and the sTNFRs are released by the host during meningitis in an attempt to control the inflammatory response.

Several types of anti-inflammatory drugs have been shown to be advantageous in the treatment of *experimental* bacterial meningitis; these include corticosteroids, anti-cytokine antibodies, PAF antagonists, free radical scavengers, NOS inhibitors, and antagonists of leukocyte-endothelial cell adhesions (reviewed in Pfister and Scheld 1997). However, many of these studies did not include antibiotic treatment in addition to the anti-inflammatory therapy. *Clinically*, only **corticosteroids** have been tested as adjunctive therapy in bacterial meningitis. The current evidence on the use of dexamethasone as adjunctive therapy in the treatment of bacterial meningitis is reviewed by Townsend and Scheld (1996). There is a wide variation in the effects of dexamethasone on inflammatory indices and outcome. Nevertheless, meta-analysis (combined analysis of data from different studies) of data from four well designed double-blinded placebo-controlled studies supports the contention that neurological sequelae (particularly severe bilateral sensorineural hearing loss) are reduced by concomitant therapy with dexamethasone. Since the large majority of data is from children, the effects of dexamethasone therapy on adults is not clear. In these four studies, gastrointestinal bleeding in two patients was the only reported adverse effect. However,

since anti-inflammatories may attenuate the increased permeability of the BBB during bacterial meningitis, several groups have investigated the effect of dexamethasone treatment on antibiotic penetration of the CSF in experimental meningitis. Despite some reductions in the concentrations of antibiotics in the CSF, there appears to be no reduction in sterilisation of the CSF by most of the commonly used antibiotics (Rodriguez, Kaplan, Hawkins *et al.* 1991a; Scheld and Brodeur 1983). However, bacterial killing by vancomycin in the CSF was significantly reduced by concomitant therapy with dexamethasone (Paris, Hickley, Uscher *et al.* 1994). Therefore, the current evidence supports the use of dexamethasone therapy in the treatment of bacterial meningitis in children, but not if the choice of antibiotics includes vancomycin (i.e. for multi-resistant strains). In the case of bacterial meningitis in adults, the use of dexamethasone is advisable if the prognosis is poor, or there are already many bacteria in the CSF upon admission (Townsend and Scheld 1996). In all patients for whom dexamethasone is used as adjunctive therapy, monitoring for adverse effects (e.g. gastrointestinal bleeding) should be carried out (Townsend and Scheld 1996). In addition, it may be wise to carry out a second lumbar puncture in the treatment of multiple resistant strains to document the action of the antibiotic when adjunctive dexamethasone therapy is used.

1.1.5. Bacterial Virulence Factors

Virulence factors are those microbial elements which enhance ability to cause infection and disease. In addition to counteracting host defences, they are responsible for many of the complications of bacterial meningitis by their potent activation of the inflammatory response.

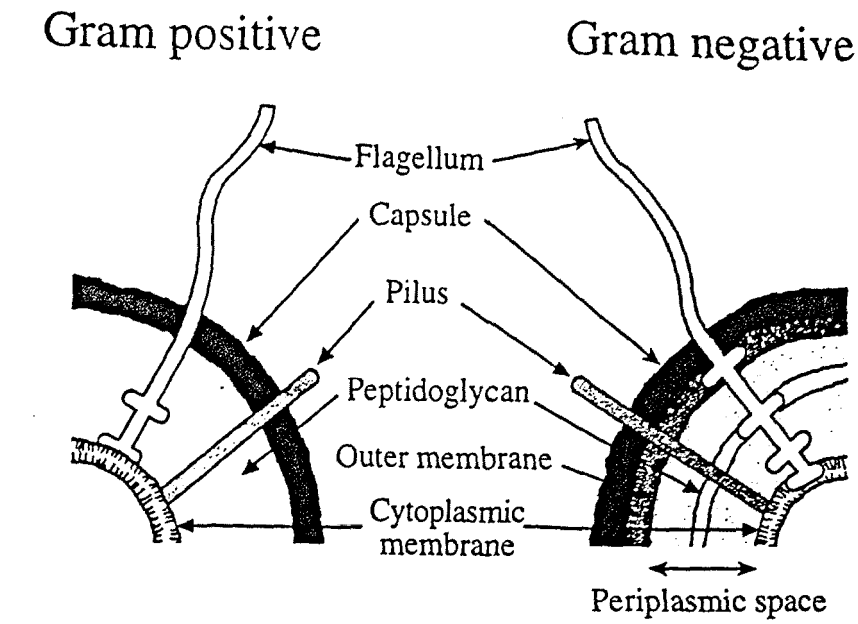
Surface encapsulation is an important virulence attribute in evading host defences. All the common meningitogenic bacteria are encapsulated. Moreover, bacterial meningitis is most commonly caused by a small proportion of the numerous capsular types associated with each meningeal pathogen. Since unencapsulated strains are often more adherent and invasive than encapsulated strains *in vitro* (St Geme and Falkow 1992), it would seem that the capsule does not facilitate translocation across cellular barriers. The prevalence of encapsulated strains is more likely due to the ability of the capsule to enhance survival in the blood and CSF by inhibiting

neutrophil phagocytosis and resisting classical complement-mediated bactericidal activity. The fact that virulence of meningitogenic bacteria is determined mainly by the capsule is demonstrated by the highly protective action of specific anti-capsular antibodies in human and experimental meningitis caused by *H. influenzae* type b, *N. meningitidis* and *S. pneumoniae* (Greenwood, Slack and Peutherer 1997).

Cell wall components are considered to be the most important bacterial factors in evoking an inflammatory response. The cell wall of *gram positive* bacteria is comprised mostly of peptidoglycan and lipoteichoic acid (LTA), whereas the cell wall of *gram negative* bacteria contains only a single layer of peptidoglycan, but also contains endotoxin (Lipopolysaccharide); **(Fig. 1.1.5.)**.

Lipopolysaccharide (LPS) is found embedded in the outside layer of the outer membrane of all gram negative bacteria. It is the one of the most significant virulence structures in gram negative bacteria. LPS is excreted by bacterial cells in small amounts, but can produce toxicity while still attached to the bacterial cell wall (Mustafa, Ramillo, Syrogiannopoulos *et al.* 1989c). Since toxicity evoked by LPS can be blocked by neutralising the lipid component (Lipid A) (Burroughs, Cabellos, Prasad *et al.* 1992; Tauber, Shibl, Hackbarth *et al.* 1987), it follows that this is the toxiphore in this complex molecule. The polysaccharide component has a variable terminal segment exposed on the outer surface of the bacteria which is a major surface antigen. The LPS of *H. influenzae* type b and *N. meningitidis* has very short polysaccharides and hence is conventionally called lipooligosaccharide (LOS). Hib and meningococcal LOS show heterogeneity both within and between strains (Roche and Moxon 1995; Smith, Parsons and Cole 1995). They each can be modified *in vivo* and become capable of resistance to LOS directed antibodies. In the case of *H. influenzae* type b, this modification is serum dependent but the mechanism is unknown (Inzana and Anderson 1985). In the case of *N. meningitidis* this is due to sialylation of terminal sugar residues which leads to the generation of an epitope that mimics one present on host cells. *N. meningitidis* can sialylate its LOS endogenously, or exogenously from substrates in *in vivo* secretions, serum and host cells (Smith *et al.* 1995). Some strains of *H. influenzae* type b have endogenously sialylated LOS components (Mandrell, McLaughlin, Aba

a)



b)

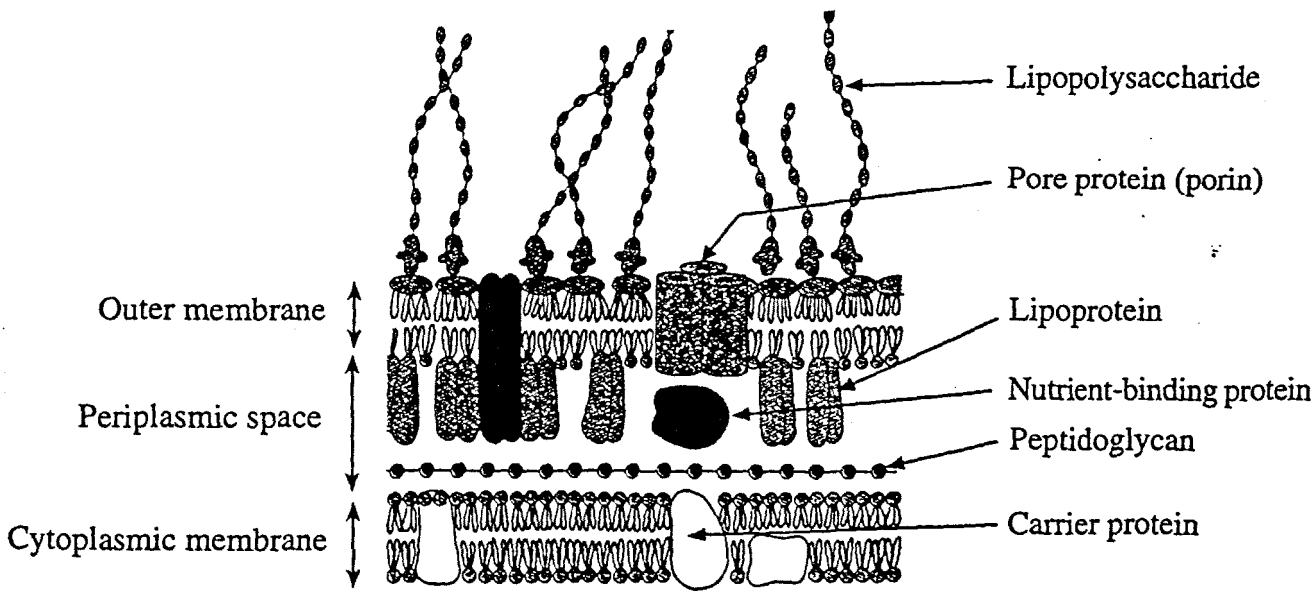


Figure.1.1.5. a) Comparison of the cell envelope structures of gram-positive and gram-negative bacteria.

b) Molecular structure of the gram-negative cell envelope.

Taken from Medical Microbiology, Murray, Drew, Kobayashi and Thompson.
The C.V. Mosby Company 1990.

Kwiak *et al.* 1992), but their involvement in the pathogenesis of *H. influenzae* type b is not known. *H. influenzae* type b LOS has been shown to be involved in a number of the pathological events that occur during meningitis and alone has been shown to evoke responses identical to the events evoked by living bacteria (Burroughs *et al.* 1992). *H. influenzae* type b LOS inhibits ciliary activity of human respiratory epithelial cells *in vitro* (Stephens and Farley 1991), and facilitates dissemination of *H. influenzae* from the nasopharynx to the blood *in vivo* (Weiser, Williams and Moxon 1990). In addition, *H. influenzae* type b LOS causes a dose-dependent and time-dependent increase in permeability of the BBB with a greater effect than live *H. influenzae* type b despite identical LPS concentrations *in vivo*. Since meningeal inflammation produced by LPS is significantly reduced by inhibition of leukocytosis (Tuomanen *et al.* 1989) it is thought that the majority of the BBB damage produced by LOS *in vivo* is mediated by the leukocytosis evoked by LPS. However, Hib LOS evokes breakdown of an *in vitro* BBB model (monolayers of cultured bovine brain endothelial cells) via a serum-dependent cytotoxic pathway (Patrick, Betts, Frey *et al.* 1992). Therefore, Hib LOS appears to be capable of some cytotoxic effects which are not secondary to leukocytosis *in vitro*. Neutralisation of meningococcal LOS in experimental sepsis enhanced survival slightly (Bannatayne, Harnett and Cheung 1977), but the role of meningococcal LOS in meningitis has not been studied. Meningococcal LOS was toxic in fallopian tube organ culture, but did not affect ciliary function in nasopharyngeal organ cultures (Stephens and Farley 1991). However, since meningococcal LOS can be modified by sialylation exogenously as well as endogenously, *in vitro* effects may not fully represent *in vivo* effects.

Pneumococcal cell walls which contain considerable amounts of **peptidoglycan** and **cell wall polysaccharides** including **teichoic acid (TA)**, are also capable of inducing an inflammatory response which is very similar to that produced by live pneumococci. In the rabbit model of bacterial meningitis, pneumococcal teichoic acid and peptidoglycan, (but not capsular material) at physiologically relevant concentrations evoked meningeal inflammation (measured by CSF white blood cell (WBC) count) similar to that seen after infection with whole pneumococci (Tuomanen *et al.* 1985). Since purified pneumococcal cell wall material is a powerful stimulus for the production of IL-1 by human monocytes (Riesenfeld-Orn *et al.* 1989), this cytokine most likely mediates many of the inflammatory effects produced by pneumococcal cell wall. Like

LPS, peptidoglycan is released in a cell-free form during growth and particularly following death induced by cytokines (Burroughs, Prasad, Cabellos *et al.* 1993). Purified peptidoglycan from *H. influenzae* induced meningeal inflammation in a rabbit model of meningitis (Burroughs *et al.* 1993). Thus it would appear that in this context peptidoglycan is involved in both gram negative and gram positive bacterial virulence. There is some evidence that antibodies directed towards cell wall polysaccharides are protective in animal models of pneumococcal meningitis (Briles, Forman, Horowitz *et al.* 1989). However, these antibodies do not appear to stimulate phagocytosis, and are much less protective than anti-capsular antibodies. In addition, the levels of anti-cell wall polysaccharide antibodies were not significantly different in healthy subjects of different ages, or between healthy subjects and patients with pneumococcal disease (Musher, Watson and Baughn 1990). Therefore the relative contribution made by antibodies to pneumococcal cell wall components to overall host defence mechanisms is not clear.

Many meningitogenic bacteria produce **IgA1 proteases** which may facilitate colonisation of mucosal surfaces by reducing opsonisation by secretory IgA1. *H. influenzae* type b, *N. meningitidis* and *S. pneumoniae* all produce IgA1 specific proteases (Kornfeld and Plaut 1981). However, no direct evidence for their role in pathogenicity exists because they are highly specific and do not cleave IgA from any of the animals used in experimental meningitis.

Pili are filamentous protein structures composed of repeating identical subunits (pilins). *H. influenzae* type b and *N. meningitidis* express pili, and are capable of pili phase variation, which is environmentally regulated (Stull, Mendelman, Schoenborn *et al.* 1984; Pinner, Spellman and Stephens 1991). To my knowledge, there is no evidence that pili are involved in the pathogenesis of pneumococcal infections. Hib pili may enhance adherence to human epithelial cells in culture (St Geme and Cutter 1995), but were not essential for adherence to human epithelial cells in organ culture (Stephens and Farley 1991). Moreover, non piliated Hib were more invasive in organ culture (Stephens and Farley 1991). Therefore Hib pili may be advantageous for colonisation, but are not essential for invasion. Since the majority of Hib isolates from the blood and CSF of patients with meningitis lack detectable pili (Stull *et al.* 1984), it appears that following colonisation and invasion of the nasopharynx, pili are not needed by Hib.

In contrast, pili are important virulence factors in meningococcal infections. Expression of assembled pili appears to be the major determinant of meningococcal attachment to nasopharyngeal epithelial cells (Stephens and Farley 1991). In addition, meningococcal isolates from the blood and CSF are very often piliated (De Voe and Gilchrist 1975), therefore pili may play a role in meningococcal virulence beyond initial colonisation and invasion (Stephens and Farley 1991). Meningococcal pili appear to mediate interactions with endothelial cells, epithelial cells and erythrocytes (reviewed in Nassif and So 1995). Therefore pili could be involved in adhesion to cerebrovascular epithelial cells in order to facilitate invasion of the CSF (as seen by *E.coli*) (Parkkinen, Korhonen and Pere 1988). In addition, since pili-deficient mutants of meningococci are not able to haemagglutinate erythrocytes (Nassif and So 1995), pili may facilitate haemagglutination and therefore promote thrombosis.

Exotoxins are protein toxins secreted from bacterial cells during growth, or released upon lysis, which can promote damaging effects in parts of the body remote from the site of infection. Some of the exotoxins which may play a role in virulence of *S. pneumoniae, H. influenzae* type b and *N. meningitidis* are discussed below.

Autolysin is a major cell-wall hydrolase which enhances virulence of pneumococci by producing autolysis. Autolysin is thought to be bound to lipoteichoic acid in the pneumococcal cell envelope (Diaz, Garcia, Acaso *et al.* 1989). If pneumococcal cell wall biosynthesis ceases (e.g. due to nutrient starvation or antibiotic treatment) autolysin is activated and causes the cell to lyse. Studies using autolysin-deficient pneumococcal mutants have provided conflicting evidence concerning the contribution of autolysin to pneumococcal virulence (reviewed in Paton, Andrew, Boulnois and Mitchell 1993). However, immunisation with autolysin was only effective against bacteria which are capable of expressing pneumolysin, and the degree of protection was similar to that produced by immunisation with pneumolysin (Lock, Hansman and Paton 1992). Therefore autolysin appears to mediate its virulence predominantly by release of pneumolysin.

Pneumolysin is a thiol-activated cytolysin released upon lysis of pneumococci. It is produced by

virtually all clinical isolates of *S. pneumoniae* (Kanclerski and Mollby 1987) and appears to be highly conserved amongst pneumococcal serotypes (Mitchell, Mendez, Paton *et al.* 1990). Significant titres of antibodies directed against pneumolysin are found in patients with pneumococcal disease, and are low in healthy subjects within the highest risk ages groups (Kanclerski, Blomquist, Granstrom *et al.* 1988). At high concentrations, pneumolysin oligomers form transmembrane pores in mammalian cells which leads to cell lysis. At lower (sublytic) concentrations, pneumolysin is toxic to virtually every type of cell in the lung (Cundell, Masure and Tuomanen. 1995), stimulates the production of many inflammatory cytokines (Houldsworth, Andrew and Mitchell 1994), inhibits the respiratory burst, bactericidal activity and migration of human PMNs (Paton and Ferrante 1983), inhibits antibody synthesis (Ferrante, Rowan-Kelly and Paton 1984), and activates the classical complement pathway in the absence of anti-toxin antibody (Paton, Rowan-Kelly and Ferrrante 1984). The role which pneumolysin plays in the pathogenesis of pneumococcal infections has been clearly demonstrated by reduced virulence in pneumolysin deficient mutants (Berry, Yother, Briles *et al.* 1989; Berry, Paton and Hansman 1992). In addition immunisation with pneumolysin significantly improved survival of mice to subsequent pneumococcal challenge infections (Paton, Lock and Hansman. 1983).

A **second haemolysin** produced by S. pnuemoniae has recently been demonstrated. Haemolytic activity previously attributed to pneumolysin has now been demonstrated with pneumolysin deficient mutants and with wild type S. pneumoniae treated with anti-pneumolysin antisera (Canvin, Paton, Boulnois *et al.* 1997). The role that this second haemolysin plays in pathogenesis has not yet been determined.

Neuraminidase is an enzyme produced by all clinical isolates of *S. pneumoniae* (O'Toole, Goode and Howe 1971) which cleaves sialic acid from host glycolipids, glycoproteins and oligosaccharides on cell surfaces or in body fluids. The incidence of coma and bacteraemia is significantly greater in patients with elevated CSF levels of neuraminidase (O'Toole *et al.* 1971). Immunisation of mice with neuraminidase slightly enhanced their survival times in subsequent pneumococcal challenge infections (Lock, Paton and Hansman 1988). Preliminary data from studies using neuraminidase-deficient mutant pneumococci did not provide any evidence of a role

for neuraminidase in the virulence of pneumococci, however further investigations are necessary (Winter 1997). Neuraminidase is capable of causing considerable damage and may also aid adhesion to epithelial and endothelial cells by exposing cell surface receptors (Paton *et al.* 1993).

Hyaluronidase cleaves hyaluronic acid and is produced by virtually all clinical isolates of *S. pneumoniae* (Brooks *et al.* 1995). Preliminary findings using hyaluronidase-deficient mutant pneumococci suggest that hyaluronidase may play a role in the virulence of pneumococci, however further investigations are necessary (Winter 1997). Since hyaluronic acid is found in connective tissue and extracellular matrices it could be proposed that hyaluronidase aids bacterial colonisation and dissemination.

Haemocin is a bactericidal protein produced by 95% *H. influenzae* type b strains which has been strongly associated with encapsulated *H. influenzae* type b strains (Tunkel and Scheld 1993) and may play a role in the pathogenesis of invasive *H. influenzae* type b infections, particularly in the initial colonisation of the nasopharynx (LiPuma, Sharetzsky, Edlind and Stull 1992).

Two iron-regulated proteins related to the RTX (repeats in toxin) family of toxins have recently been described in meningococci (Thompson, Wang, West *et al.* 1993). Proteins belonging to the RTX family have a common gene sequence of amino acid repeats arranged in tandem and are usually related to target specific cytolytic exotoxins produced by gram negative bacteria. The function of the RTX related proteins in *N. meningitidis* is not yet known, although they appear to be non-haemolytic and non-leukotoxic (Thompson *et al.* 1993).

1.1.6. Host defences

In order to counteract invading organisms, the mammalian host has developed a number of defence mechanisms.

IgA antibodies are present in nasopharyngeal secretions and serum. Many meningitogenic bacteria including *H. influenzae* type b, *N. meningitidis* and *S. pneumoniae* produce IgA specific

proteases which cleave serum and secretory IgA antibodies (Kornfeld and Plaut 1981). However, some of the IgA antibodies (e.g. IgA2 subclass) are resistant to hydrolysis by bacterial proteases and so may play a role in preventing colonisation (Gilbert, Plaut, Longmaid *et al.* 1983).

Within the host, bacteria are phagocytosed by **leukocytes** facilitated by **antibodies** and the **complement system**. Complement and antibody activity in the CSF is raised only slightly (if at all) during meningitis, and is insufficient for bacterial lysis or opsonisation (Simberkoff, Moldover and Rahal 1980) therefore blood borne phagocytosis is the site of most bacterial killing. In the non-immune host opsonisation for phagocytosis can only be achieved by complement activity. In the immune host the combination of antibody and complement activity can be used for opsonisation. Some antibodies (e.g. against *H. influenzae* type b capsule) can act as opsonins independent of complement activity, but full opsonic potential is only achieved when the classical complement pathway is activated. Therefore complement activity is essential for opsonisation of encapsulated meningitogenic bacteria such as *H. influenzae* type b, *N. meningitidis* and *S. pneumoniae* in both immune and non immune patients. This has been illustrated clinically, because a greater incidence of bacterial meningitis is seen in individuals with compromised complement systems (Ross and Densen 1984). However, different components of the complement system appear to be important in the elimination of individual pathogens. For example, opsonisation by C3b appears to be particularly important in *H. influenzae* type b infections (Noel, Bauer, and Edelson 1992) whereas C5-C8 bactericidal activity is particularly important in meningococcal disease (Densen 1989). An outline of the complement cascades can be seen in **Figure 1.16**.

Of all types of antibodies, those directed towards capsular polysaccharides are the most protective against meningitogenic bacteria including *H. influenzae* type b, *S. pneumoniae* and several serotypes of *N. meningitidis*. However, most capsular polysaccharides elicit T-cell independent responses, i.e. there is no induction of memory, the response is oligoclonal, dependent on age, lacks affinity maturation and has poor antibody class switching. Therefore, in addition to capsular polysaccharides, vaccines against meningitogenic bacteria incorporate other somatic antigens which are T cell dependent in order to induce a more effective antibody

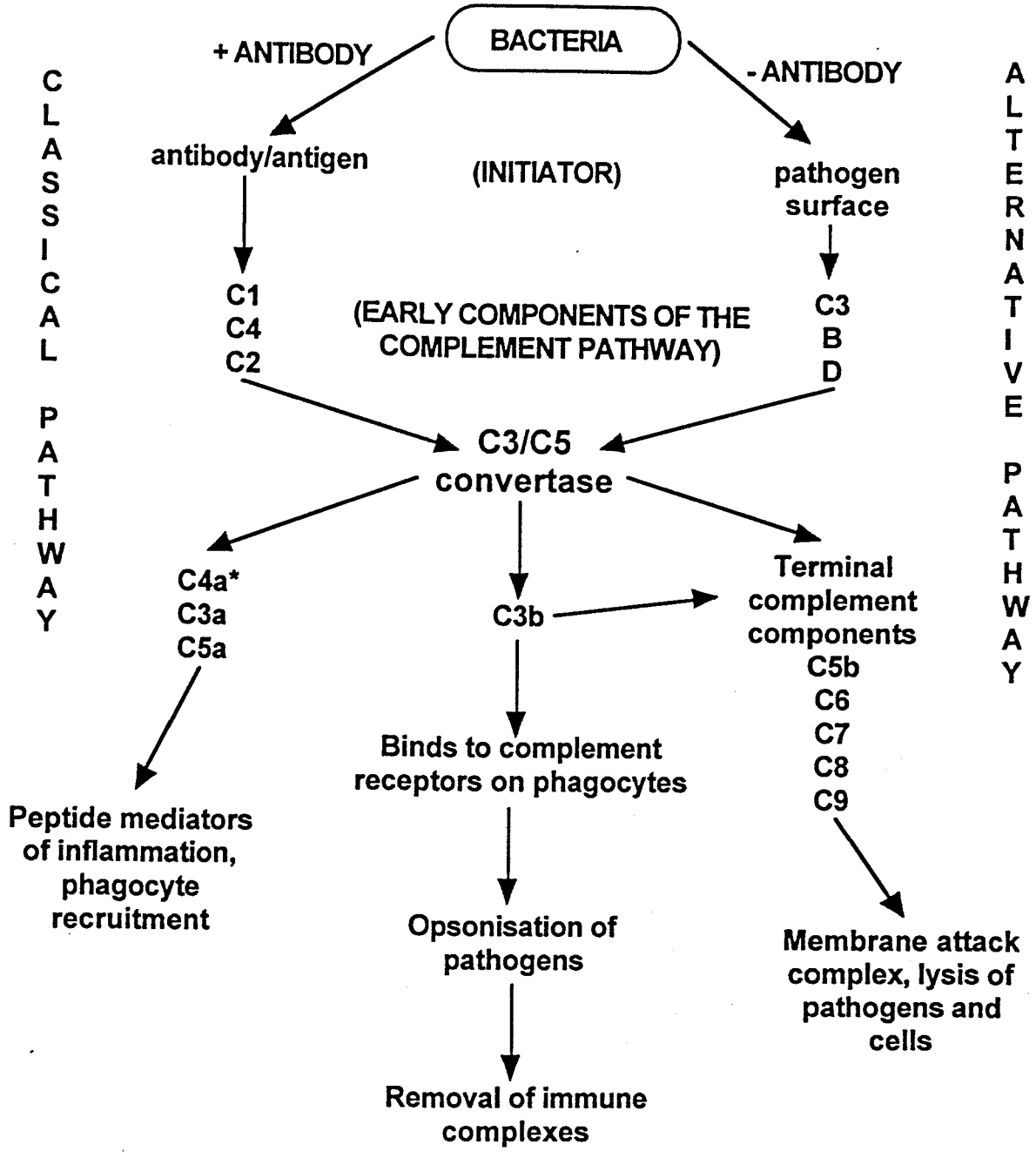


Figure 1.16. Overview of the main components and effector actions of complement (Taken from Janeway and Travers 1994).

The early events of both the classical or antibody-initiated pathway and the alternative pathway involve a series of cleavage reactions culminating in the formation of an enzymatic activity called a C3/C5 convertase, since it cleaves complement components C3 and C5. This is the point at which the two pathways converge and the effector functions of complement are generated. The larger cleavage fragment of C3 (C3b) binds to the membrane and opsonizes bacteria, allowing phagocytes to internalise them. The small fragments of C5 and C3 called C5a and C3a, are peptide mediators of local inflammation. C4a, generated by cleavage of C4 during the early events of the classical pathway (* and not by the action of C3/C5 convertase) is also a peptide mediator of inflammation, but its effects are relatively weak. Similarly, the large cleavage fragment of C4, C4b is a weak opsonin (not shown). Finally, C3b binds C5, allowing generation of C5b that associates with the bacterial membrane and triggers the late events, in which the terminal components of complement assemble into a membrane-attack complex that damages the membrane of the pathogen.

response which is independent of age and induces anamnestic responses. Anti-Hib capsular and anti-pneumococcal capsular antibodies develop in most children by the age of 3-4, however anti pneumococcal capsular antibodies decline past the age of 50-60. Therefore those most at risk from *H. influenzae* type b and pneumococcal disease are those aged <3-4 and >50 (pneumococcal disease only)

***H. influenzae* type b vaccines** have been introduced world wide and have resulted in a dramatic fall in the incidence of *H. influenzae* type b disease. There are several conjugate vaccines available which consist of the polyribosylribitolphosphate (PRP) polysaccharide from type b capsule covalently linked to a protein (e.g. diphtheria and tetanus toxoids) which enhances the immunogenicity of PRP.

Currently a 23-valent polysaccharide **pneumococcal vaccine** is available which contains capsular polysaccharides from types 1, 2, 3, 6B, 9N, 9V, 10A, 11A, 12F, 14, 15b, 17F, 18C , 19F, 19A, 20, 22F, 23F and 33F. However, there are two problems with this vaccine. Firstly the vaccine is not protective against all isolates (90%), and secondly, the vaccine is poorly immunogenic in those most at risk (e.g. immunocompromised patients, elderly people and young children (Greenwood *et al.* 1997; Musher 1992). Therefore the vaccine is most commonly given to patients with predisposing factors such as those with sickle cell anaemia or those who have undergone splenectomy (Greenwood *et al.* 1997).

Meningococcal vaccines containing the polysaccharides of groups A, C, Y and W135 are available and are currently used in selective populations (e.g. military and civilian epidemics). However, the vaccine provides only short-lasting protection and the group C polysaccharide is not immunogenic in infants less than 2 years old (Lepow, Goldschneider, Gold *et al.* 1977; Ceesay, Allen, Menon *et al.* 1993). There is as yet no vaccine for *N. meningitidis* type B: the group B polysaccharide is poorly immunogenic in humans of all ages.

1.2. Hearing loss in Bacterial Meningitis.

It is thought that bacterial meningitis is responsible for 90% of profound hearing impairments acquired postnatally in children under 3 (Fortnum 1992). Fortnum (1992) reported that between 1977-1990 the incidence of sensorineural deafness in bacterial meningitis was between 3.5 and 37.2% with a mean incidence of 9.6%. The incidence of hearing loss varies according to the bacterial pathogen. Pneumococcal meningitis is associated with the greatest incidence of hearing loss followed by Hib and meningococcal infections respectively. Fortnum (1992) reported that between 21.4 and 50% (mean of 31.8%) patients with pneumococcal meningitis develop sensorineural hearing losses compared with 5.9-18.1% (mean of 11.4%) of patients with Hib meningitis and 5-10.5% (mean of 7.5%) with meningococcal meningitis.

Several methods have been adopted to detect hearing loss during human and experimental meningitis. Before describing these methods an outline of the anatomy and physiology of the cochlea is given.

1.2.1. Anatomy of the Cochlea

The inner ear contains the vestibular and auditory sense organs. The **cochlea** (auditory apparatus) is a coiled bony channel within the petrous part of the temporal bone. It is 35mm long with $2^1/_4$ turns in man. The cochlea contains the auditory receptor cells and is divided longitudinally into three chambers (**Fig.1.2.1.1.**). The inner most central chamber is called *scala media*. **Scala media** does not communicate with the other scalae and contains the sensory organ of Corti. It is filled with *endolymph* which is very similar to intracellular fluid (high $[K^+]$ and low $[Na^+]$). Scala media is separated from an upper compartment called *scala vestibuli* by Reissner's membrane, and a lower compartment called *scala tympani* by the basilar membrane. At the base of the cochlea **scala tympani** and **scala vestibuli** are bordered by membranes designated round and oval windows respectively. At the apex of the cochlea these two scalae meet at a small opening called helicotrema. These peripheral chambers are filled with *perilymph* which resembles an ultra filtrate of the CSF (high $[Na^+]$ and low $[K^+]$). At the base of the cochlea, there is a small opening

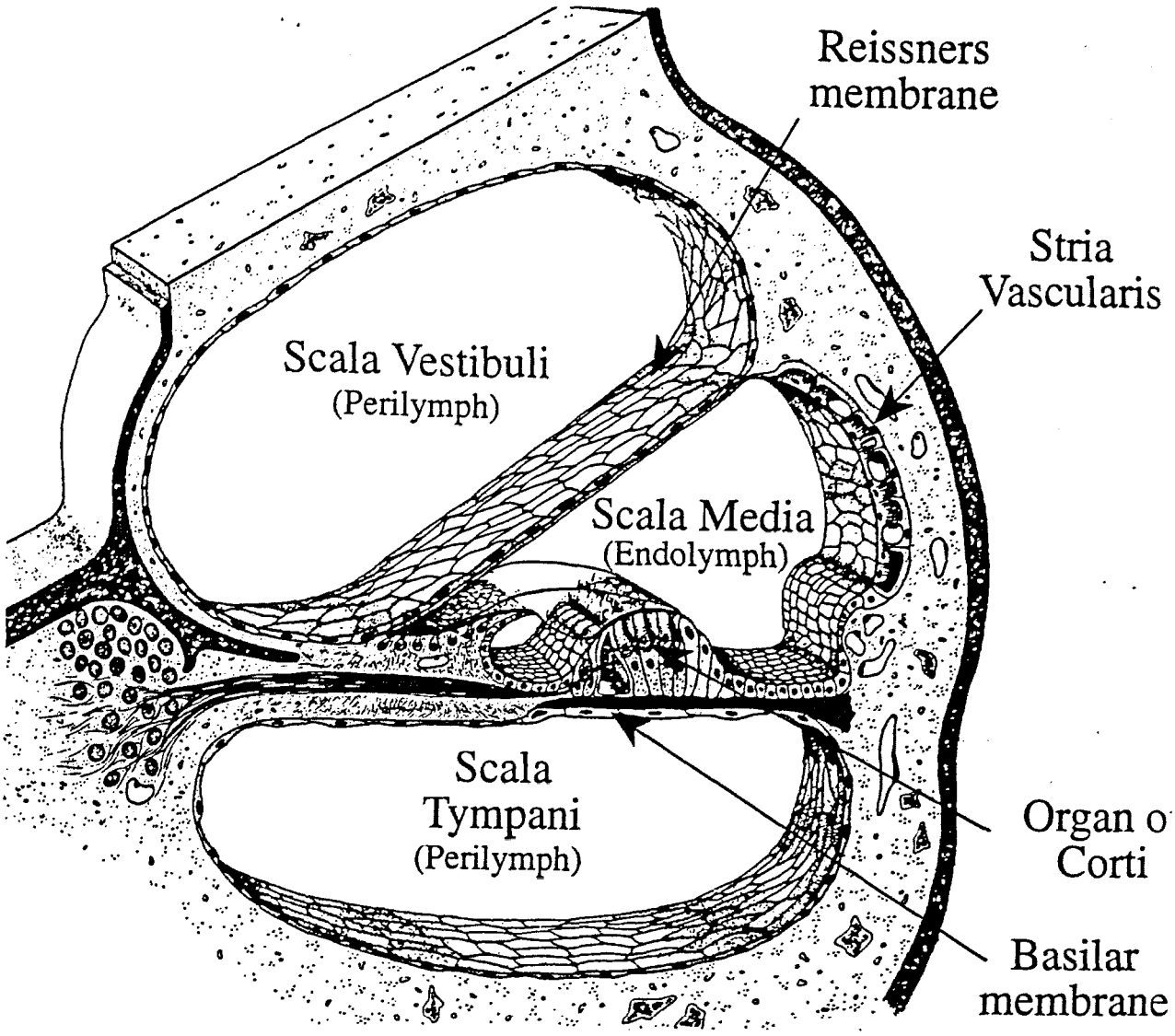


Figure 1.2.1.1. A section through one of the turns of the cochlea.
(Taken from Guyton 1991 Textbook of Medical Physiology 8th Edition)

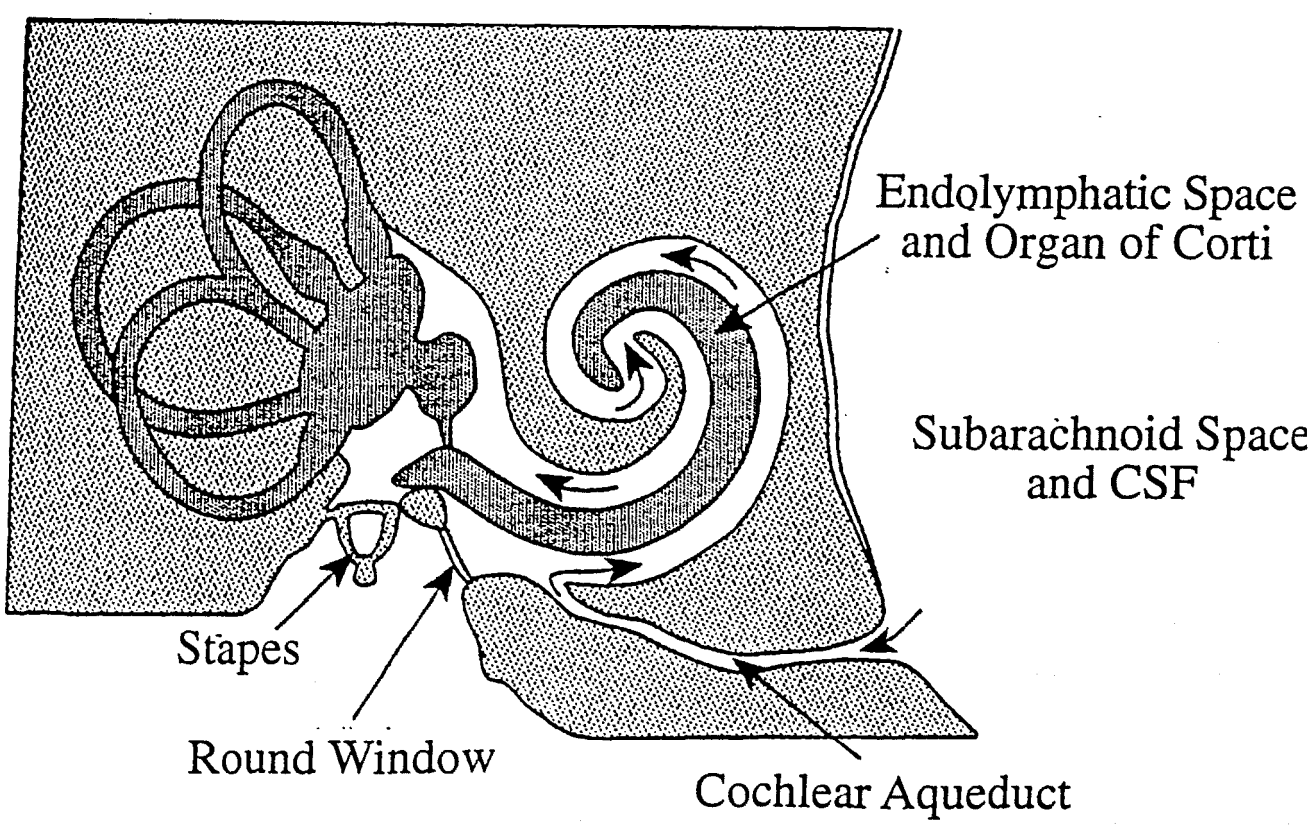


Figure 1.2.1.2. A cross section through the temporal bone to illustrate the connection of the CSF with the perilymph of the cochlea via the cochlear aqueduct. (Taken from Bhatt *et al.* 1993)

in the wall of scala tympani which leads into the *cochlear aqueduct* which connects the CSF in the sub arachnoid space with the perilymph of the cochlea (**Figure 1.2.1.2.**). The central axis of the cochlea is the bony modiolus which contains the vascular and neural elements which serve the cochlea. Lining the outer wall of scala media a three-cell-layered structure is found which is called *stria vascularis*. **Stria vascularis** contains an intra-epithelial capillary plexus and secretory cells. Stria vascularis which is the major blood supply of the cochlea and is also responsible for the maintenance of the large positive endocochlear potential within scala media (+80mV) by actively pumping ions in and out of the endolymph.

The **organ of Corti** is a specialised structure containing the sensory *hair cells* which rests on the basilar membrane (**Fig.1.2.1.3.**). It consists of one row of inner hair cells and three rows of outer hair cells. The inner hair cells and outer hair cells are separated by a triangular Tunnel of Corti formed from pillars/rods of Corti which give rigidity to the structure. The inner hair cell and outer hair cell populations lie medial and lateral to the Tunnel of Corti respectively. Supporting cells surround the rods and hair cells. The supporting cells and rods of Corti hold the organ of Corti firmly on top of the basilar membrane. Cuticular thickenings at the apex of hair cells (*cuticular plates*), tight junctions between the hair cells and supporting cells, and phalangeal processes from supporting cells form a *reticular lamina* which precludes the perilymph and endolymph from mixing. This produces an electrochemical gradient across the hair cells because the surface of the cells are surrounded by endolymph whereas the rest of the cell is bathed in perilymph. Lying above the hair cells is the tectorial membrane. The *tectorial membrane* is a gelatinous and fibrous flap fixed to the Organ of Corti only on its inner radial edge.

Hair cells are sensory cells adapted to transduce mechanical stimuli to electrical signals. The mechanosensitive organelle, the *hair bundle*, is found at the apical surface of the hair cells inserted into the cuticular plate. The **hair bundle** contains 3-4 rows of 30-300 specialised processes named *stereocilia*. The hair bundle is hexagonally packed and stereocilia are arranged in ranks of increasing height. **Stereocilia** are rigid structures whose cytoskeleton is composed of hexagonally packed bundles of longitudinal actin filaments, which are extensively cross-linked (Tilney, Derosier and Mulroy 1980). The stereocilia taper a few μm above the basal insertion into

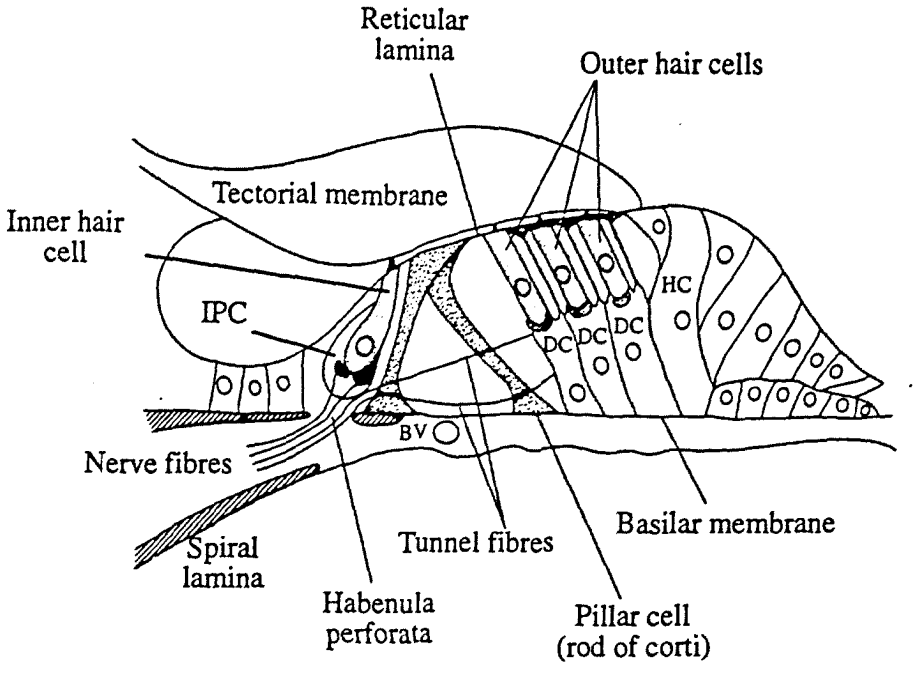


Figure 1.2.1.3. A cross section through the organ of Corti as it appears in the basal turn. BV: Blood Vessel; DC : Deiters' cells; HC : Hensen's cells; IPC : Inner phalangeal cell. Taken From Pickles 1982.

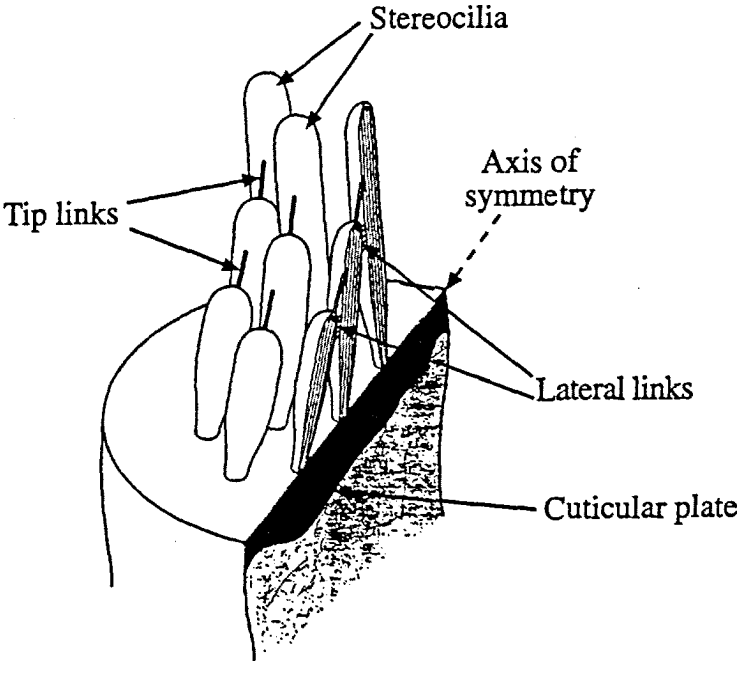


Figure 1.2.1.4. Representative diagram of a cross section through a mammalian hair cell to illustrate the organisation of the stereocilia and links connecting them. Adapted from Pickles and Corey 1992.

the cuticular plate to form a rootlet at which point the stereocilia pivot and slide along one another's shafts (Tilney *et al.* 1980). Lateral links run parallel to the apical surface of the hair cell joining adjacent stereocilia in all directions (Osborne and Comis and Pickles 1984). They interconnect stereocilia to allow them to pivot most easily along an axis which is 90° to mirror symmetry. There are also specialised links called *tip links* at the tips of the stereocilia which join the top of each stereocilia to the side wall of adjacent taller stereocilia (**Fig. 1.2.1.4.**). **Tip links** have a fine central filament likely to be proteinaceous (Osborne and Comis 1990) surrounded by a glycocalyx (Osborne, Comis and Pickles 1988). The ability of tip links to stretch by greater than 100%, their diameter of 6nm, and, the fact that they disappear after treatment with elastase all point to an elastin-like component of tip links (Preyer, Hemmert, Zenner, and Gummer 1995; Osborne and Comis 1990).

Outer hair cells are cylindrical shaped cells with a W or V shaped arrangement of stereocilia whereas **inner hair cells** are flask shaped cells with a line arrangement of stereocilia. The tips of the tallest stereocilia of the outer hair cells but not the inner hair cells are firmly embedded within the tectorial membrane (Lim 1980).

1.2.2. Mechanism of hearing

Sound waves are directed into the middle ear by the external ear and cause the tympanic membrane to vibrate. This auditory signal at the tympanic membrane is transferred via the ossicles to the oval window of the cochlea. The sound energy forces the oval window in and out of scala vestibuli producing pressure waves within the perilymph. The wave of pressure passes from the base to the apex of the cochlea in scala vestibuli, and then passes out of the cochlea along the basilar membrane and scala tympani. It is the displacement of the basilar membrane that stimulates the organ of Corti where the mechanical energy is transduced into an electrical signal (**Fig. 1.2.2.1.**). The tectorial membrane and basilar membrane are hinged on different axes and the travelling wave motion of the fluids and structures within the cochlea causes a shearing force between the basilar membrane and the tectorial membrane. This shearing force directly deflects the hair bundles of the outer hair cells as the stereocilia are embedded within the tectorial

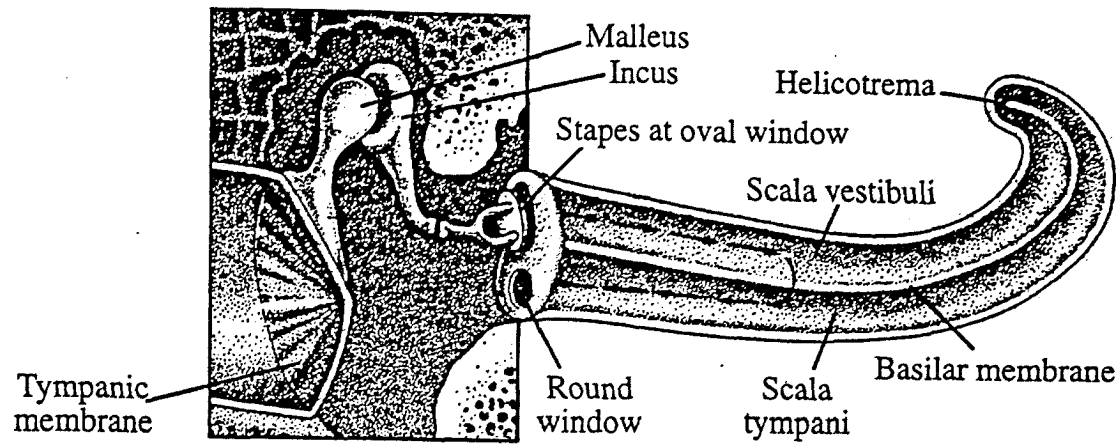


Figure 1.2.2.1. Longitudinal section through the cochlea and middle ear to illustrate the passage of sound energy. Taken From Berne and Levy 1996.

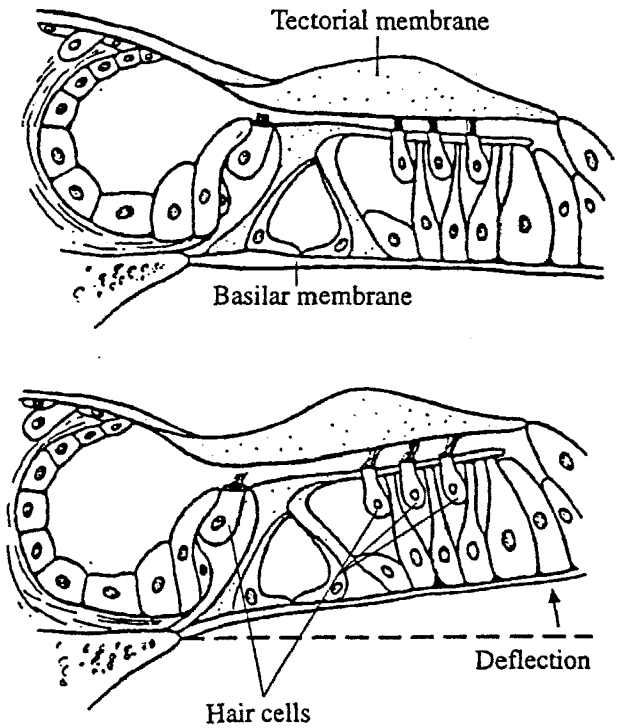


Figure 1.2.2.2. Cross section through the organ of Corti to show the shearing force between the tectorial membrane and basilar membrane during sound stimulation. This shearing force directly deflects the hair bundles of the outer hair cells as the stereocilia are embedded within the tectorial membrane, and indirectly deflects the hair bundles of the inner hair cells by the subsequent flow of sub-tectorial fluid. Taken From Berne and Levy 1996.

membrane, and indirectly deflects the hair bundles of the inner hair cells by the subsequent flow of sub-tectorial fluid (**Fig 1.2.2.2.**). Hence inner hair cells are velocity sensitive and outer hair cells are displacement sensitive.

Deflection of the hair bundle in a positive direction (towards its taller edge) causes depolarisation of the hair cell (*receptor potential*). If the **receptor potential** increases, the rate of neurotransmitter release at the basal surface is increased. Displacement of the hair bundle in a negative direction (towards the shorter stereocilia) hyperpolarises the hair cell, reducing the receptor potential and hence reducing the rate of release of neurotransmitter. Oblique deflection of the hair bundle evokes a response dependent on the vector component of the stimulus in the positive direction. The receptor potential is dependent on both direction and magnitude of the stimulus. The relationship between displacement and the receptor potential is sigmoidal hence the response saturates at a certain point (**Fig. 1.2.2.3.**). The relationship is very steep such that 90% response is evoked by deflection of the stereocilia by only 50-120nm at the tip. The depolarisation and hyperpolarisation are achieved by the opening and closing of ionic transduction channels respectively. In the unstimulated cell 15% transduction channels are open hence the number of channels open can be increased or decreased. The resting potential of the cell is approximately -60mV which is the result of the inward flow of cations through the 15% transduction channels open (reviewed in Hudspeth 1989).

The transduction channels are relatively non selective to alkali cations (Li^+, Na^+, K^+, Rb^+ & Cs^+ are roughly equally permeant: Corey and Hudspeth 1979a). Since endolymph is rich in K^+, these ions carry much of the transducer current. Ca^{++} is however a necessary cofactor in transduction since transduction ceases below about 10μM Ca^{++} (Corey and Hudspeth 1979a). The increased receptor potential evoked by the influx of cations opens voltage gated Ca^{++} channels in the hair cell membrane, and the resultant increase in the influx of Ca^{++} evokes an increase in the rate of release of transmitter into the synaptic cleft. The increase in transmitter binding to receptors on the post-synaptic membrane results in an increased depolarisation of the afferent nerve ending. The increased depolarisation of the post-synaptic membrane increases the rate of action potentials

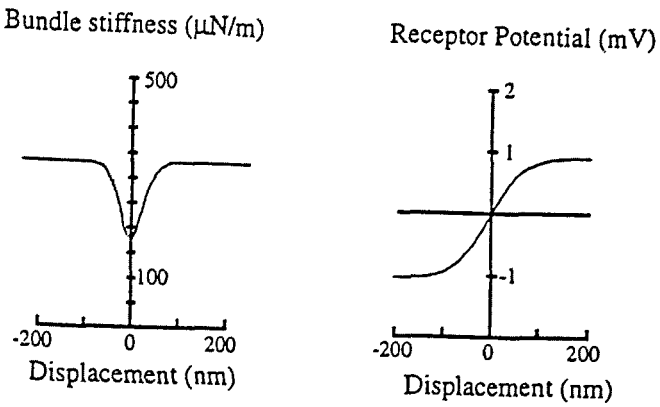


Figure 1.2.2.3. Graphs to illustrate the range of movements over which the hair cell shows greatest a) compliance, and b) receptor potential. Gating compliance is a reduction in the hair bundle stiffness (a) measured by applying known forces to a bundle with a flexible glass fibre, then calculating from the fibres flexion the stiffness of the attached bundle. The fact that the compliance is greatest over the range of hair bundle positions where transduction is most sensitive (b) suggests that the compliance results from the opening and closing of transduction channels.
(Taken from Hudspeth 1989)

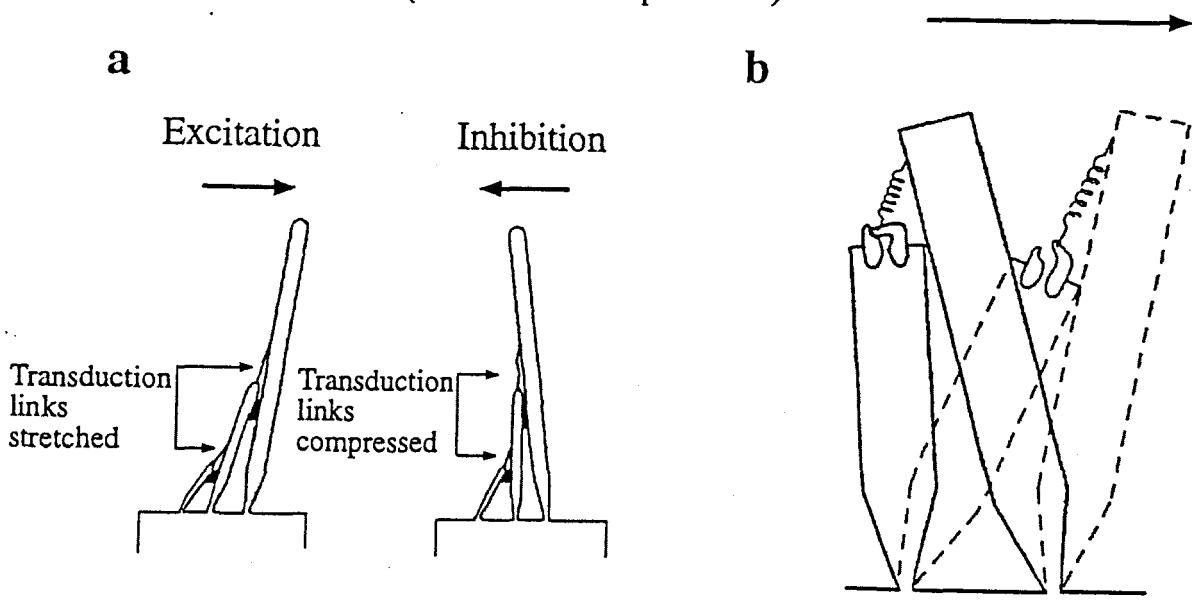


Figure 1.2.2.4. Diagrams to illustrate the tip link/gating springs theory of transduction proposed by Pickles *et al.* 1984.
a) The lateral links hold the tips of the shorter stereocilia against the longer stereocilia. When the bundle of stereocilia is deflected, a vertical shear is produced between the rows, which is detected by the single upwards–pointing tip link (transduction link), running from the tip of the shorter stereocilium. It is suggested that the stretch of the this tip link decreases the membrane resistance. Taken from Pickles *et al.* 1984.
b) A simple structural model comes from the suggestion that each tip link is a gating spring. Displacing the bundle towards the tallest stereocilia (a positive stimulus) stretches the tip links to increase tension. The channels (somewhere in the distal ends of the stereocilia) are shown in this figure to be at the lower end of each tip link, but equally well could be at the upper end, or, indeed both. Taken from Pickles and Corey 1992.

sent along the auditory nerve to the cochlear nucleus. Movement in the negative direction closes these transduction channels, decreasing K^+ and Ca^{++} entry and thus hyperpolarising the cell. This leads to a decrease in the excitatory neurotransmitter release and hence a decrease in activity in the afferent neurone.

The link between hair bundle deflection and transduction channel opening was the missing link in the mechanotransduction theory. The short latency of the response (approximately 13μs at 37°C in bullfrog sacculus hair cells) and temperature dependence (Corey and Hudspeth 1979b) led these investigators to believe that a simple direct coupling system was most likely. Therefore they proposed a biophysical model in which the transduction channels were directly gated by an elastic element (Corey and Hudspeth 1983). Deflection of stereocilia in a positive direction puts tension on an elastic element (which had not yet been identified) which directly opens transduction channels allowing cation flow and hence depolarising the cell. Movement in the opposite direction reduces the tension on the elastic element directly closing transduction channels and hyperpolarising the cell by reducing cation flow into the cell. This model was supported by measurements of hair bundle compliance which were greatest over the range of bundle displacements at which transduction channels open and close (Howard and Hudspeth 1988). This had been predicted by the gating spring model due to the shortening of the elastic gating elements when the channels are moved from their closed to open state. The identification of tip links by Osborne, Comis and Pickles 1984 then lead to the tip link theory of transduction (Pickles, Comis and Osborne 1984) which has become widely accepted (other possible explanations are reviewed by Hackney and Furness 1995 and Pickles and Corey 1992). It is based on a gating spring hypothesis in which the elastic element is the tip link (**Figs. 1.2.2.4. and 1.2.2.5.**) The main lines of evidence are:

- The tip link is the only known structural element which can explain the directional specificity of the sensitivity of the hair cells.
- Tip links appear to be present in all vertebrate hair cells capable of mechanotransduction (reviewed in Pickles and Corey 1992).
- Most evidence suggests that transduction channels are located at the tips of stereocilia which is consistent with the location of tip links (reviewed in Denk, Holt, Shepherd *et al.* 1995 and

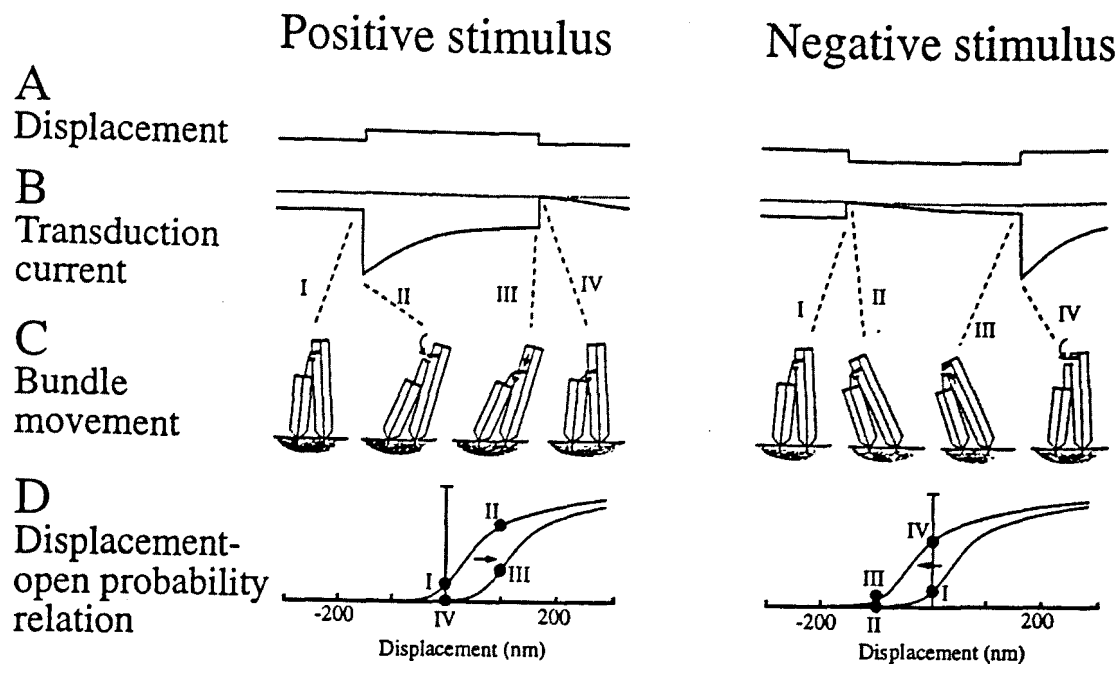


Figure 1.2.2.5. Gating spring model of transduction and adaptation.
A depicts a positive (left) and negative (right) deflection of a hair bundle (direction indicated in C) **B** indicates the resultant transduction current (top line is zero). A positive stimulus increases the inward transduction current (left), as adaptation proceeds, the current declines toward a plateau. During negative stimulation, the transduction current (right) initially declines then rebounds as adaptation occurs. **C** depicts the proposed tip link theory of transduction and adaptation. Positive stimulation (left) elongates the tip link stretched between the stereocilia and thus pulls open a transduction channel. The entry of Ca^{++} into the cytoplasm provokes slipping of the myosin molecules at the insertional plaque, so that the tip link shortens and the channel recloses. When a negative stimulus is presented (right) molecules pull the tip link's insertion up the stereocilium, restoring tension and reopening the channel. **D** indicates the displacement-response relations. When a bundle sits at its resting position, transduction channels carry about 15% of their maximal current (point I at left). The responses that occur during rapid deflections of the bundle to other positions (for example, point II) define a sigmoidal relation between bundle displacement and the channels' open probability. As adaptation takes place this sigmoidal relationship is shifted along the displacement axis. Adapted from Hudspeth and Gillespie 1994

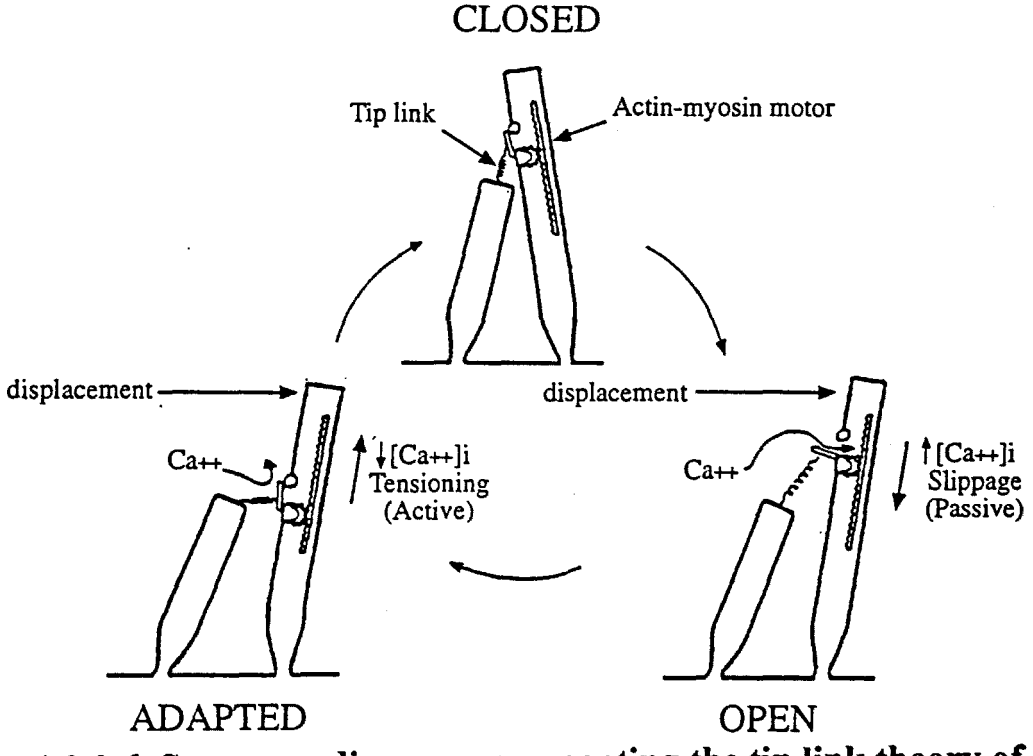


Figure 1.2.2.6. Summary diagram representing the tip link theory of adaptation. When stimulated, opening of transduction channels allows Ca^{++} entry into the stereocilia at the tip link. This causes passive slipping of the tip link down an actin-myosin motor closing the transduction channel. If moved in the opposite direction adaptation occurs by active tensioning of the tip link by moving up the actin/myosin motor. Taken from Hamill and McBride 1994.

Hackney and Furness 1995).

- Elimination of extracellular Ca^{++} which inhibits transduction is also associated with a positive displacement of the hair bundle (the stereocilia appear to relax forward) and the tip links appear to vanish (Assad *et al.* 1991).
- Estimates of the number of transduction channels per cell have been derived from several physiological measurements and are equal to 1 or two per stereocilia which is consistent with transduction channels at one or both ends of the tip link (Hackney and Furness 1995).
- The tip link theory of transduction provides an explanation for adaptation (see next section).

In order to respond to small stimuli despite background inputs there is a need for resetting of responsiveness by the hair cells **(adaptation)**. Steady displacement of a hair cell bundle causes the displacement/voltage curve to move along the displacement axis to match the new position of the bundle without any appreciable change in shape of the curve (Howard and Hudspeth 1988). It is an active mechanism performed by the hair cells which reduces the response to saturating displacements and repositions the maximum sensitivity of the steady bundle position. Adaptation is best explained by the tip link theory for mechanotransduction. The most recent theory of adaptation is that a molecular motor at the upper end of the tip links regulates tension in the tip links by sliding up and down the actin core of the stereocilium **(Fig. 1.2.2.6.)**. The rate of slipping down or climbing up is regulated by Ca^{++} concentration which is determined in part by Ca^{++} influx through transduction channels (reviewed in Hudspeth and Gillespie 1994). The motor is thought to be a cluster of approximately 50 myosin molecules, most evidence suggests myosin I or a related myosin isozyme. Calmodulin appears to be important in binding to and controlling activity of the hair bundle myosin I (Walker and Hudspeth 1996).

The cochlea is capable of fine **frequency tuning**. Each site along the cochlea is maximally sensitive to a particular frequency, and each site projects fibres to a specific site in the cochlear nucleus (tonotopic organisation). The base of the cochlea responds to high frequency auditory stimuli, whereas the apex of the cochlea responds to low frequency stimuli. A number of *passive* and *active* mechanisms are involved. I shall briefly outline these mechanisms.

Passive tuning is achieved by the varied structure of the cochlea from the base to the apex. The resonant frequency of any object is determined by its mass and its flexibility. The shape and composition of the basilar membrane is not homogeneous along its length, hair cells are shorter at the base than at the apex. The stereocilial bundles of the hair cells also vary along the length of the cochlea, stereocilia being longer and less stiff at the apical end of the cochlea and shorter and stiffer at the basal end of the cochlea. This gradient in the structure of the organ of Corti along the cochlea results in maximum displacement of the basilar membrane and hair bundles at a specific site along the cochlea according to the frequency of the stimulus. Although passive tuning is responsible for some of the frequency tuning ability of the cochlea, it cannot fully explain the sharp tuning characteristics of the cochlea. Therefore additional **active tuning** mechanisms are involved *in vivo*. Mathematical models suggest that there could be some source of mechanical power which simultaneously overcomes the viscous damping and provides the filtering and sensitivity seen in the cochlea (reviewed in Yates, Johnstone, Patuzzi *et al.* 1992). Outer hair cells are capable of mechanomotility, that is they can perform mechanotransduction in reverse, to convert electrical activity into movement of the hair cell (Brownell, Bader, Bertrand *et al.* 1985). In addition, outer hair cell activity is essential for maximal frequency tuning responses in the cochlea (reviewed in Pickles 1985). Therefore it is thought that outer hair cells are responsible for the active tuning of the cochlea through a motile response which produces a mechanical input into basilar membrane movement. Hair bundle deflection of outer hair cells produces a receptor potential which then in turn leads to a change in shape of the outer hair cell. This change in shape of the outer hair cells is thought to amplify actively the movement of the basilar membrane at this particular site. In this way the outer hair cells counter the damping effects of the cochlear fluids and increase the signal-to-noise ratio by selectively amplifying the response to a relevant frequency. The precise motile response involved in active tuning is not yet clear. An excellent review of the several different motile responses by outer hair cells which have been reported in response to both mechanical deflection and electrical stimulation is given by Yates *et al.* (1992).

1.2.3. Innervation of the cochlea

Afferent and efferent innervation of the cochlea may be divided into 2 distinct groups according to which hair cell type is innervated (**Fig. 1.2.3.**).

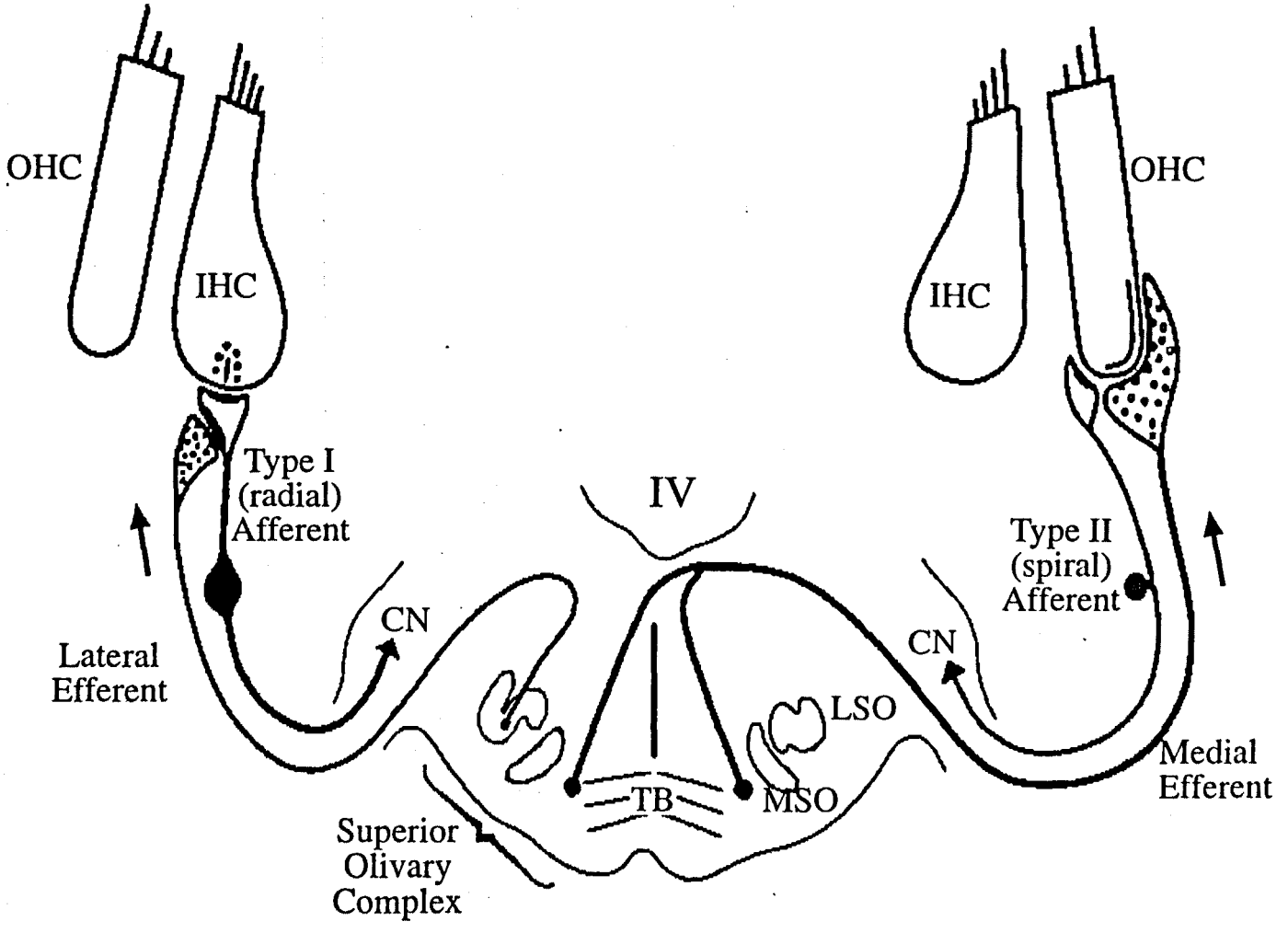


Figure 1.2.3. Schematic drawing of the neural organisation of the mammalian cochlea.
For clarifity, inner hair cell circuitry has been completed on only the left, and outer hair cell circuitry on the right.
TB: trapezoid body; MSO: medial superior olivary complex; LSO: lateral superior olivary complex;
CN: Cochlear nucleus; IV: Fourth ventricle.
Adapted from Eybalin

Afferent innervation is made up of neurones whose cell bodies are found in the spiral ganglion. **Type I** afferents receive input from inner hair cells, they make up 90-95% afferent innervation of the cochlea and each inner hair cell receives up to 30 afferent synapses. **Type II** afferents receive input from outer hair cells, they make up only 5-10% afferent input from the cochlea, and each collateral receives input from 6-100 outer hair cells. The type I neurotransmitter has been studied extensively. It is likely that more than one neurotransmitter is involved, but most evidence points towards glutamate as the principal neurotransmitter. The neurotransmitter of type II afferents has been less well studied due to the difficulty in isolating purely type II synapses. However the little data there is points towards glutamate or a parent substance operative at this synapse too (reviewed in Eybalin 1993).

There are several types of glutamate receptors named according to their most potent agonists
There are 3 main ionotropic receptors, (linked directly to ion channels) these are N-methy-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and kainic acid (kainate). There is also a metabotropic receptor which is linked to the second messenger inositol trisphosphate. The metabotropic receptor is agonised by quisqualate, as is the AMPA receptor. There is strong evidence for the presence of the three ionotropic receptors in the cochlea (Eybalin 1993). Recently it has been shown that quisqualate evokes an increase in inositol trisphosphate in the rat cochlea. This suggests that metabotropic receptors may also be present in the cochlea (Pujol, Eybalin and Puel 1995).

Efferent innervation of the cochlea originates in the superior olivary complex. **Medial efferents** synapse directly onto the base of outer hair cells. Each fibre innervates several outer hair cells often from different rows, but with most innervation on the internal row. The function of the medial efferents has been well studied and the current theory is that medial efferents contribute to the mechanical tuning which the outer hair cells provide through their motility (reviewed in Eybalin 1993). **Lateral efferents** are unmyelinated fibres which synapse onto type I afferent dendrites on their postsynaptic terminals at the base of the inner hair cells. The function of lateral efferents is less well understood. It is thought that lateral efferents may be responsible for

establishing and/or maintaining the spontaneous activity of type I fibres. There is now considerable evidence that both medial and lateral efferents utilise acetylcholine (Ach) as a neurotransmitter. However, Ach is not the only neurotransmitter involved since opioid neurotransmitters are involved (Sahley and Nodar 1994), and GABA may also be utilised (Felix and Ehrenberger 1992).

1.2.4. Methods of assessing hearing loss

Several methods have been employed to detect meningitis-induced hearing losses in human clinical cases and experimental animals. In all cases, conductive deafness associated with otitis media should be precluded by examination of the tympanic membrane and tympanometry.

Conventional pure tone audiometry is used clinically in patients who have recovered, and are old enough (usually children >3-4 years old) to produce reliable subjective measurements. In neonates and younger children or during acute illness in older subjects and in experimental meningitis hearing is most commonly assessed by **auditory brain stem evoked responses** (ABERs). By coupling electrodes to the skin at the vertex of the skull and to the mastoid on both sides, brain stem electrical potentials can be recorded. The response to several 100s or 1000s of transient auditory stimuli is averaged which eliminates random activity and sums activity produced in response to the auditory stimuli. The resultant waveform (ABER) typically has 4 or 5 major peaks. Experiments in cats have shown that the WAVE I arises from the auditory nerve, WAVE II from the cochlear nucleus, WAVE III from the superior olivary complex, and WAVES IV and V from the region of the lateral lemniscus or inferior colliculus (Buchwald and Huang 1975). Analysis of these waves to various intensities of sound can give an indication of the hearing ability of the individual and an indication as to whether the hearing loss is of central (cochlear nucleus) or peripheral (i.e. cranial nerve or cochlear) origin. Recently, **otoacoustic emissions** (OAEs) have been measured in addition to ABERs in order to give an indication of the cochlear function, in patients with meningitis (Richardson *et al.* 1997). OAEs are minute sounds produced by the cochlea that can be recorded from nearly all normal ears. They are thought to represent outer hair cell activity and so can give an indication of the function of the organ of Corti. OAEs are abolished by lesions of the cochlea, but remain intact in retrocochlear deafness

(Probst, Lonsbury-Martin and Martin 1991).

In experimental models of meningitis, the ABER method has been the commonest method of hearing assessment. However, more recently **auditory evoked cochlear potentials** have been recorded from the round window membrane during experimental meningitis in the guinea pig (Winter *et al.* 1997). An electrode lying on the round window membrane (or inserted into scala tympani) can pick up electrical potentials produced by the cochlea in response to sound stimuli. The ***compound action potential*** (CAP) is the most frequently recorded cochlear potential because it is a very reliable and informative response. It arises from the synchronous discharge of a large number of afferent fibres in the auditory nerve. It is a postsynaptic response produced by the afferent nerve fibres innervating the region of the cochlea most sensitive to a particular stimulus frequency (**Figure 1.2.4.**). The CAP is best elicited by stimuli that have a short rise time (<1ms) such as an acoustic click or tone. Since travelling waves travel most rapidly over the basal part of the cochlea, only fibres from the high frequency basal region of the cochlea are activated in synchrony. Therefore cochlear fibres responding to 4kHz and above are the main contributors to the gross CAP (Pickles 1982). By recording CAP responses to different frequencies a measurement of the ability of the cochlea to respond to sounds of different frequencies can be compared before, during and after experimental meningitis. The size of the CAP is affected by all the events that lead to afferent neurone activity, that is conduction of sound to the inner ear, transduction by the hair cells and synaptic transmission. The CAP can also be affected by efferent activity.

Another commonly recorded cochlear potential which can be obtained from the round window membrane (or from within scala tympani) is the **cochlear microphonic** (CM). The CM is the alternating electrical response (receptor potential) produced by hair cells during an acoustic stimulus which exactly mirrors the stimulus in terms of frequency and phase. The CM is elicited by pure tones of moderate intensity. Therefore the CM is a good indicator of hair cell function. Since outer hair cells comprise approximately $^{3}/_{4}$ of the hair cell population, they are the main contributors to the CM. The CM is affected by all events that lead to production of a receptor potential in the hair cells, that is conduction of the sound to the inner hair cells and

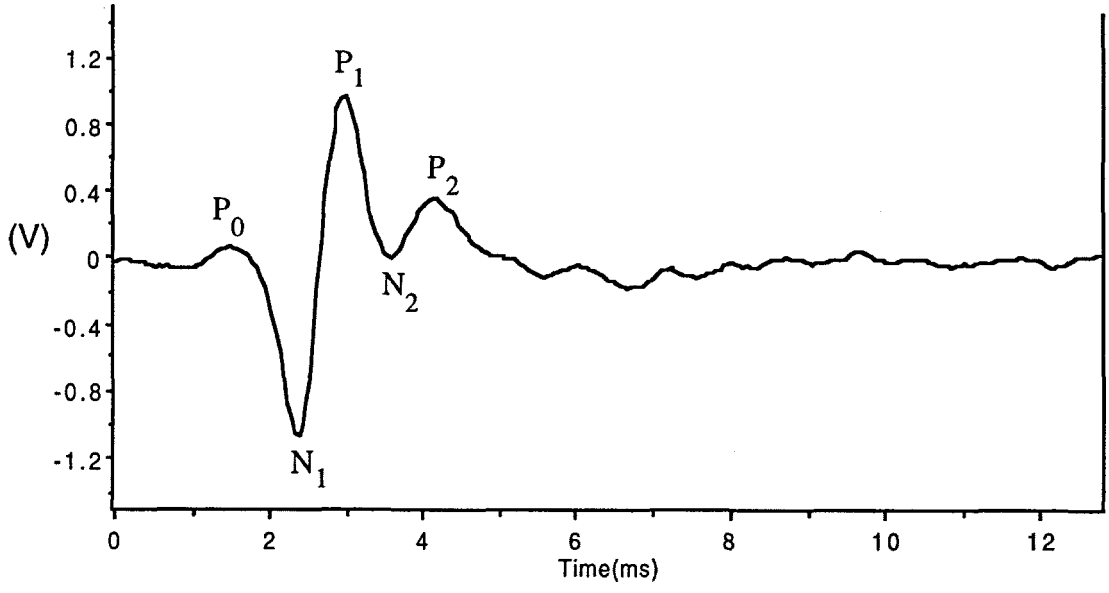


Figure 1.2.4. Example of a Compound Action Potential (CAP) elicited with a 10kHz tone pip (amplified 20,000x).
The peaks and troughs of the wave form (positive and negative deflections) are denoted by a P and N respectively, and numbered in sequence.

mechanotransduction by the hair cells.

1.2.5. Site of damage responsible for hearing loss in Meningitis

Sensorineural hearing loss associated with meningitis could be a result of damage to one or more sites: central auditory nuclei and fibres, peripheral auditory neurones (i.e. eighth cranial nerve), or the inner ear itself. However, several lines of evidence indicate that the majority of meningitis-induced hearing losses result from damage to the cochlea itself. The first clinical evidence for this was from post mortem examinations of temporal bones from patients who had died from bacterial meningitis. These examinations revealed labyrinthitis, i.e. inflammation of the inner ear (Igrashi, Saito, Alford *et al.* 1974; Eavey, Gao, Schuknecht *et al.* 1985). This was first confirmed in experimental meningitis by Moxon *et al.* (1974). Intranasal inoculation of *H. influenzae* type b into infant rats produced labyrinthitis (as judged by post mortem histological examinations) in several animals. Labyrinthitis has now been demonstrated following experimental meningitis in several species and induced by several bacteria, including Hib in infant rats (Moxon *et al.* 1974 ; Kaplan *et al.* 1989), adult rats (Wiederman *et al.* 1986) and adult rabbits (Osborne *et al.* 1995), *S. pneumoniae* in infant rats (Rodriguez, Kaplan, Hawkins *et al.* 1991b), adult guinea pigs (Blank, Davis, VanDeWater *et al.* 1994; Winter *et al.* 1997) and adult rabbits (Bhatt *et al.* 1991; Bhatt, lauretano, Cabellos *et al.* 1993), *Streptococcus suis* in adult guinea pigs (Kay 1991), and *Escherichia coli* in adult rabbits (Osborne *et al.* 1995). However, cochlear histology has not been reported in meningitis induced by *N. meningitidis*.

Clinically, the next major step in identifying the lesion responsible for meningitis-induced deafness was the development of the ABERs which allowed assessment of the hearing ability in patients with acute meningitis. This technique has revealed that meningitis-induced hearing losses are early in onset (<24 h admission to hospital), and late onset hearing losses do not occur (Vienny, Despland, Lutschg *et al.* 1984). In addition, the hearing losses are likely to have peripheral origin in many cases since they can develop in the absence of apparent brain damage (Ozdamar, Kraus and Stein 1983). Transient hearing losses have also been reported in patients

with bacterial meningitis (Vienny *et al.* 1984). The first experimental model of meningitis to incorporate hearing assessment was carried out by Kay (1991). Measurement of ABERs in response to click stimuli in guinea pigs inoculated with *S.suis* produced hearing losses in some animals which were associated with labyrinthitis. This was also seen in an adult rabbit model of pneumococcal meningitis (Bhatt *et al.* 1991); hearing loss developed in all animals, and began at approximately 12 h post inoculation which confirmed the early onset of meningitis-induced hearing losses. In addition, Bhatt *et al.* 1991 used tone evoked ABERs and found that hearing losses progressed from high to low frequencies, consistent with a basal to apical spread of inflammation. Morphological examinations by electron microscopy of cochleae following similar experiments but induced by *E.coli* revealed specific lesions to the organ of Corti associated with meningitis-induced hearing losses (Winter, Marwick, Osborne *et al.* 1996). Finally, Winter (*et al.* 1997 or more detailed in Winter 1997) developed an adult guinea pig model of pneumococcal meningitis which allowed the recording of cochlear potentials from the round window membrane of the cochlea. This model confirmed that the cochlea was the site of meningitis-induced deafness since pneumococcal meningitis consistently produced CAP losses equivalent to more than 20dB at 6 h post intracranial inoculation, which as judged by EM examinations were associated with specific lesions to the organ of Corti.

All the histological studies of the cochlea following human and experimental meningitis discussed here were concerned with inflammation predominantly in the perilymph of cochleae; there were little of no inflammatory cells in the endolymph (Igrashi *et al.* 1974; Eavey *et al.* 1985; Moxon *et al.* 1974; Wiederman *et al.* 1986; Kaplan *et al.* 1989; Rodriguez *et al.* 1991b; Kay 1991; Bhatt *et al.* 1991; Bhatt *et al.* 1993; Blank *et al.* 1994; Osborne *et al.* 1995; Winter *et al.* 1997). Since the perilymphatic chambers of the cochlea are directly connected to the CSF by the cochlear aqueduct, it would seem most likely that bacteria gain access to the cochlea through this route. In fact, inflammation and bacteria were often found in the cochlear aqueduct following human and experimental meningitis (Igrashi *et al.* 1974; Eavey *et al.* 1985; Moxon *et al.* 1974; Wiederman *et al.* 1986; Bhatt *et al.* 1991; Bhatt *et al.* 1993; Blank *et al.* 1994). The basal-apical progression of both hearing losses and inflammation described by Bhatt *et al.* 1993 is consistent with a cochlear aqueduct route of entry. The cochlear aqueduct contains a meshwork of reticular

cells and connective fibres among which are occasional macrophages. Almost all human cochlea aqueducts (96-100%) are patent and large enough for the passage of both bacteria and inflammatory cells (Palva 1970; Rask-Anderson, Stahle and Wilbrand 1977). Therefore this is an obvious route by which bacteria could enter the cochlea. The other alternatives are a hematogenous spread or a modiolar route of entry for which there is little evidence following experimental meningitis. However, a recent temporal bone study from patients with acute meningitis revealed that in addition to the cochlear aqueduct, the modiolus is likely to be a common and important pathway in humans by its perineural and perivascular channels (Merchant and Gopen 1996). Merchant and Gopen (1996) concluded that the modiolus may be a more common route of infection in humans than in rabbits, possibly due to the anatomical differences of these structures between the two species.

1.2.6. Mechanism of cochlear damage during bacterial meningitis.

The mechanism by which cochlear damage occurs during meningitis is not clear. In order to assess whether bacterial factors or inflammatory mediators are ototoxic, two main methods have been employed. In both cases, test substances are administered into scala tympani at the base of the cochlea whence it diffuses across the basilar membrane into the organ of Corti. This administration site is close to the cochlear aqueduct and so closely resembles entry of bacterial/inflammatory factors during meningitis. The test substance is either perfused directly into scala tympani by making a small hole in the outer bone of the cochlea, or applied to the window through which it diffuses into scala tympani. Hearing can then be assessed by either ABERs, OAEs, or by recording cochlear potentials such as the CAP and CM (described in section 1.2.4.). Additional measurements include the endocochlear potential (described in section 1.2.1) which is essential for mechanoelectrical transduction, leading to electrical activity of single fibres of the auditory nerve. Employing the round window and cochlear perfusion methods, some bacterial factors have been shown to produce ototoxicity in the cochlea.

1.2.6.1. Bacterial Ototoxins (toxins which cause ototoxicity)

S. pneumoniae

Comis, Osborne, Stephen *et al.* 1993 showed that perfusion of either 1 or 10μg pneumolysin into scala tympani of guinea pig cochleae produced large CAP and CM losses (equivalent to >40dB) which were associated with specific lesions to the hair cells and supporting cells. More recently, Winter *et al.* (1997) found that pneumolysin-deficient mutant pneumococci produced considerably attenuated hearing losses (as judged by CAP recordings from the round window membrane) in a guinea pig model of meningitis in comparison to wild type pneumococci; much smaller hearing losses were associated with the mutant. Therefore pneumolysin is a potent ototoxin which is likely to be responsible for the majority of the hearing losses associated with this pathogen.

H. influenzae type b Ototoxins

Apart from endotoxin (see later section), no ototoxins have been isolated from *H. influenzae* type b. Preliminary unpublished data from this laboratory suggested the existence of an ototoxic protein factor in a crude cytoplasmic extract of *H. influenzae* type b. To date this is the only evidence of which I am aware of the existence of a "protein" Hib ototoxin..

Meningococcal Ototoxins

Apart from endotoxin (see next section) there is at present no evidence of an ototoxin produced by *N. meningitidis.*

In **conclusion,** despite the fact that an identified ototoxin (pneumolysin) plays a major role in the evolution of meningitis-induced deafness in experimental pneumococcal meningitis, very little work has been carried out to determine whether other potent ototoxins are responsible for the meningitis-induced hearing losses associated with other meningitogenic bacteria.

1.2.6.2. Cell wall components

Perfusion of purified **endotoxin** (LPS) (from *E.coli*, or *Salmonella typhimurium*; at 100 and 25μg

doses respectively) directly into scala tympani of guinea pig cochleae produced small losses (equivalent to <20dB) in the CAP and ABERs respectively (Comis, Osborne and Tarlow 1991; Darrow, Keithley and Harris 1992). The hearing losses were associated with a small degree of morphological damage to the surface of the organ of Corti. Maximal hearing losses produced by *E.coli* LPS were reached over a period of 4 hours, showed considerable inter animal variation, and were attenuated by prior dexamethasone treatment (Comis *et al.* 1991). The hearing losses produced by *Salmonella typhimurium* LPS were transient, peak losses were seen at days 2 and 4, but had returned to almost baseline within 7 days (Darrow *et al.* 1992). There have also been several reports of hearing losses and morphological damage to the cochlea following round window application of endotoxin purified from several different pathogens including *H. influenzae* type b (Lin 1990, Kim and Kim 1995, Watanabe and Hakuhisa 1996). Hearing losses began at around 3h, peaked at around 24-48h and persisted up to between 5 days and 2 weeks post application. Morphological examinations revealed inflammation predominantly within the perilymphatic chambers, and some hair cell damage which involved reversible vacuolation (Watanabe and Hakuhisa. 1996). Therefore, since the *in vivo* ototoxicity produced by LPS is slow in onset (several hours) and can be attenuated by prior administration of dexamethasone (Comis *et al.* 1991) it appears to be mediated by the resultant inflammatory response. However, endotoxin is directly ototoxic to outer hair cells *in vitro* (Dulon, Huang and Schacht 1990). A similar discrepancy between the *in vivo* and *in vitro* toxicity of LPS was described earlier in this chapter (section 1.1.5.) in data from experiments investigating the toxicity of LPS on the BBB. This inconsistency could be due to several reasons including *in vivo* modification of LPS, different bacterial sources of LPS, or different bioactive concentrations used.

Perfusion of 10μg lipoteichoic acid (from *Streptococcus pyrogenes*) into scala tympani of guinea pig cochleae produced a small (20-30dB) loss in the CAP at 3 h post perfusion (Unpublished data Macmillan-Browse and Comis 1994), similar to that seen by perfusion of LPS (Comis *et al.* 1991). In addition, experimental meningitis induced by pneumococcal cell wall extracts produced some small hearing losses (Winter 1997). Further investigations are clearly necessary to establish the role (if any) of these bacterial fractions in causing hearing loss.

In conclusion, release of cell wall components by autolysis and antibiotic killing during meningitis may produce a sufficient concentration of cell wall components in the CSF and/or the cochlea to be ototoxic, most likely via the resultant inflammatory response. However, such hearing losses associated with cell wall components are likely to be small and may be reversible and are therefore unlikely to be responsible for those meningitis-induced hearing losses which are profound and permanent.

1.2.6.3. Nitric Oxide, Excitatory Amino Acids and Reactive Oxygen Species

During the course of bacterial meningitis, several toxic agents are released by bacteria, leukocytes and other host cells (discussed earlier in this chapter). Amongst these products of bacterial invasion are three strong candidate mediators of ototoxicity. *Excitatory amino acids*, *nitric oxide*, and *reactive oxygen species* have all been reported to be elevated in the CSF during bacterial meningitis, and are all potentially capable of producing ototoxicity:

Several groups have now reported that perfusion of a **nitric oxide** (NO) donor (Sodium Nitroprusside (SNP)) into scala tympani of guinea pig cochleae produced a fall in the size of the auditory evoked potentials (Amaee, Comis, and Osborne 1995; Chen, Nenov, Skellett *et al.* 1995; Dean-Dais, Prazma, Ball *et al.* 1996; Kong, Ren and Nuttal 1996), including the CAP, CM, and endocochlear potential. This ototoxicity produced by SNP occurred despite an increase in cochlear blood flow (Kong *et al.* 1996) which was probably due to the potent vasodilatory action of NO. The electrophysiological losses were associated with swelling of the afferent neurones mainly on the basal side (Kong *et al.* 1996), and were mediated in part by cGMP because they were attenuated by methylene blue (Dean-Dais *et al.* 1996). The ototoxic effects of SNP were likely to have been due to its breakdown product of NO rather than cyanide, since comparable concentrations of ferrocyanide produced no ototoxicity, and preliminary data suggest that afferent swelling is also produced by pure NO donors which do not release cyanide (Kong *et al.* 1996). There are no data concerning the *in vitro* toxicity of NO in the cochlea.

Perfusion of **Reactive Oxygen Species** (ROSs) generating systems which produce H_2O_2, $OH^{\bullet}$ or

$O_2^{\bullet}$ into scala tympani of guinea pig cochleae produced some CAP losses. Since this ototoxicity was inhibited by co-perfusion with selective scavengers for each ROS (catalase, deferoxamine and superoxide dismutase respectively) the action of the generating systems was specific to ROS production (Clerici and Yang 1996). In these studies morphological examinations were not carried out. In addition, ROSs are toxic to isolated outer hair cells *in vitro* (Dulon *et al.* 1990; and Clerici, Dimartino and Prasad 1995).

Excitatory amino acids (EAAs) can produce ototoxicity in guinea pig cochleae by excessive stimulation of glutamate receptors. This has been demonstrated by both excessive release of the afferent neurotransmitter during acoustic trauma, or by perfusion with glutamate (for reviews see Eybalin 1993; Pujol, Puel, D'Aldin *et al.* 1993). The ototoxicity is selective to the afferent neurones of the inner hair cells and can be demonstrated electrophysiologically and morphologically. It is specific to the action of glutamate at the post synaptic glutamate receptors because it can be mimicked by perfusion of selective glutamate agonists. Since greatest protection on the cochlea from noise-induced and ischaemic induced excitotoxic damage was provided by inhibition of the non NMDA receptors, it can be argued that the non NMDA receptors are the predominant mediators of excitotoxicity. However, NMDA receptors are also involved because full protection also required inhibition of the NMDA receptors (Pujol, Puel and Eybalin 1992 and Pujol *et al.* 1993).

Although there have been no direct investigations into the role of NO, ROSs and glutamate in meningitis-induced deafness, a role in mediating the ototoxicity produced by pneumolysin has been implicated. Prior treatment of the cochlea with either a NOS inhibitor, or NMDA receptor antagonists protected the cochlea from the action of pneumolysin (Amaee *et al.* 1995). In addition, NMDA-evoked CAP and CM losses were blocked by inhibition of NO or the superoxide scavenger superoxide dismutase (Amaee, Comis, Osborne *et al.* 1997), and morphological damage evoked by NMDA was similar to that produced by the NO donor SNP (Amaee *et al.* 1997). In summary, ototoxicity produced by pneumolysin appears to have been mediated by NMDA receptors by production of NO and an involvement of superoxide. Since morphological examinations revealed perturbation of the reticular lamina barrier following

perfusion with pneumolysin (Amaee 1995), Amaee proposed the following scheme. Pneumolysin formed pores in the reticular lamina which resulted in mixing of the endolymph and perilymph. The raised $[K^+]$ in the perilymph resulted in excessive release of the excitatory neurotransmitter glutamate. Excessive stimulation of NMDA receptors by glutamate leads to an increase in the concentration of intracellular Ca^{++} which activated NOS to produce NO. Peroxynitrite is formed by the reaction of NO with superoxide anions and decomposes to form the highly damaging cytotoxic oxidants, hydroxyl radical and nitrogen dioxide.

Since the ototoxicity produced by the excessive release of glutamate is thought to be predominantly mediated by non-NMDA receptors (discussed earlier in this section), non-NMDA receptors could also be involved in the ototoxicity produced by pneumolysin via a common pathway. This is supported by the fact that although NMDA antagonists were capable of fully blocking all electrophysiological losses produced by pneumolysin, they were not able to completely block the morphological damage produced by pneumolysin (Amaee *et al.* 1995). There is no evidence that non-NMDA receptors mediate ototoxicity in the cochlea by NO or ROS production. However, since toxicity produced by non-NMDA receptors can be attenuated by NOS inhibitors and ROS scavengers in some brain cells (Dutrait, Culcasi, Cazevieille *et al.* 1995; Schulz, Henshaw and Siwek 1995), it is plausible. A schematic representation of the pathway by which NO and ROSs could contribute to the excitotoxicity evoked by Non-NMDA and NMDA receptor activation can be seen in **Figure 1.2.6.3**.

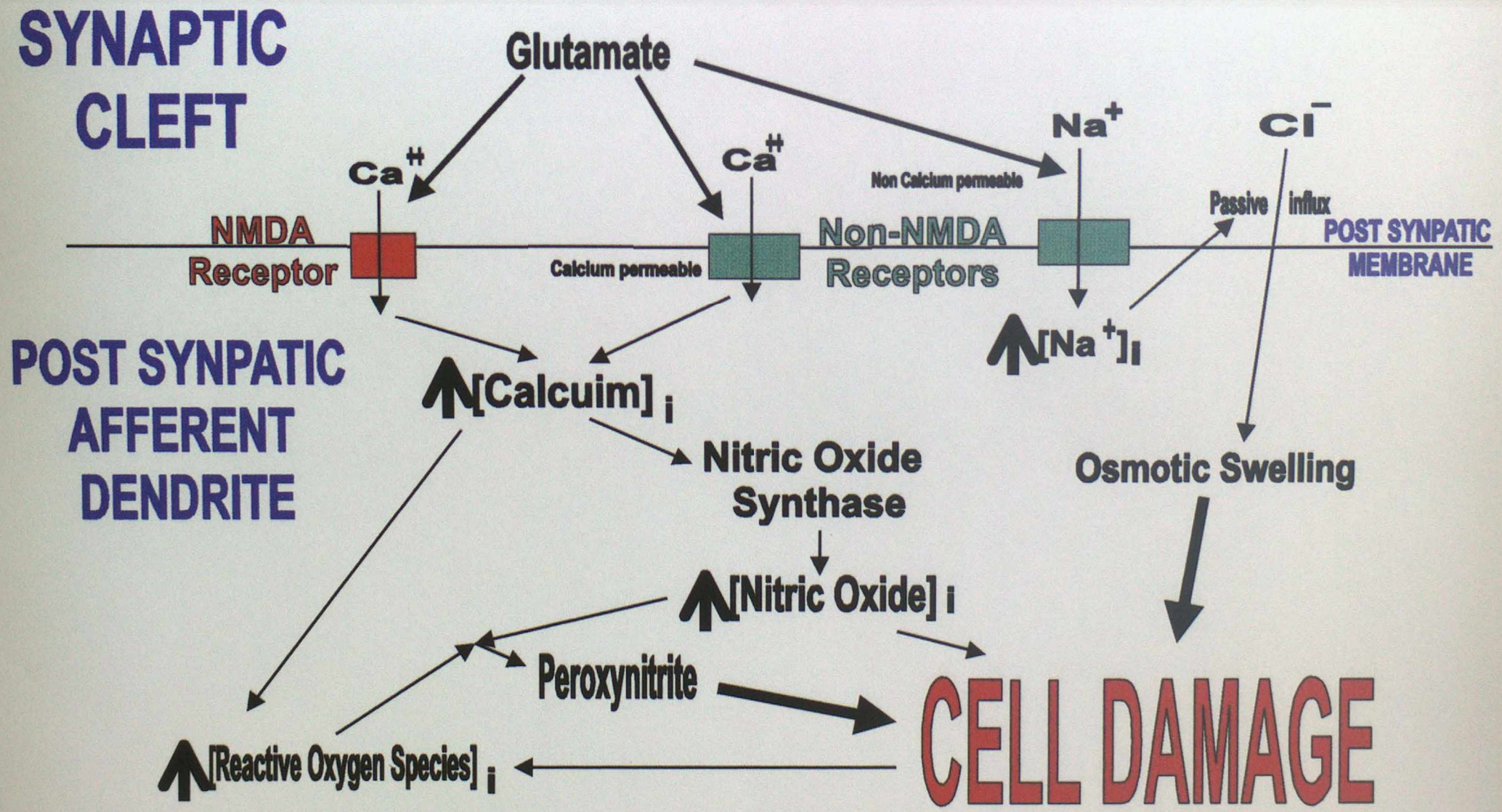


Figure 1.2.6.3. Schematic representation of the routes by which Nitric Oxide and Reactive Oxygen Species may contribute to the excititoxicity caused by Non-NMDA and NMDA receptor activation. Activation of calcium permeable Non-NMDA receptors, and activation of NMDA receptors evokes an increase in the concentration of Ca^{++} inside the post synaptic afferent dendrite. The increase in Ca^{++} could evoke cell damage in a number of ways including activation of NOS to produce NO, and release of ROSs (e.g. by inhibition of various mitochondrial enzymes). NO and ROSs alone can cause some cellular damage and if NO were to react with superoxide, peroxynitrite is produced which decomposes to form the highly damaging cytotoxic oxidants, hydroxyl radical and nitrogen dioxide. If the activation of the Non-NMDA receptors is sufficiently excessive the influx of Na^{++} (though the receptors) and Cl^{-} (passive influx) may evoke damage to the cell by osmotic swelling. [ ]i denotes intracellular concentration, and a large arrow next to this indicates in increase in intracellular concentration.

1.2.7. Hearing loss in Bacterial Meningitis : Conclusions

- The majority of meningitis-induced hearing losses originate in the cochlea, rather than the CNS.

- Bacteria and inflammatory cells gain access to the cochlea directly from the CSF and not through the blood. The most likely route of access is the cochlear aqueduct.

- Permanent meningitis-induced deafness occurs early in the course of meningitis (<12h)

- It is possible/probable that potent bacterial ototoxins are responsible for the majority of the profound meningitis-induced hearing losses.

- Less potent ototoxic factors such as cell wall components may contribute to the meningitis-induced deafness, but alone they are more likely to be the cause of small or transient hearing losses.

- Other toxic agents released during meningitis may also be involved e.g. excitatory amino acids nitric oxide and reactive oxygen species. A link between these three ototoxic agents has been proposed. However the involvement of non-NMDA receptors has not been investigated.

1.3. Aims and Objectives

1. Despite evidence that specific bacterial ototoxins are likely to be responsible for the profound permanent meningitis-induced hearing losses, there has only been one identified ototoxin isolated from the three major meningitogenic bacteria i.e. pneumolysin. Therefore the major part of this thesis is dedicated to identifying the presence of ototoxic factors in the three main meningitogenic bacteria.

2. Since NO, ROSs and excitatory acids may be involved in meningitis-induced deafness. The final part of the work described in this thesis was carried out to establish whether the action of NO and ROSs and excitotoxicity in the cochlea occurs through one common pathway. This had been demonstrated in the case of NMDA receptors, therefore this study was carried out to determine whether this is also the case with non-NMDA receptors.

CHAPTER 2

Materials and Methods

2.1 Assessment of Ototoxicity

An *in vivo* method of investigation was adopted because this most closely resembles the interaction of bacterial components and other chemicals with the cochlea during infections. The cochlear perfusion model was used as this allows us to determine whether bacterial extracts and chemicals are directly ototoxic, whereas hearing losses produced during a meningitis model may involve other routes of ototoxicity. This method mimics the route of entry during meningitis, and there is little or no barrier to diffusion of substances between scala tympani and the organ of Corti (Tonndorf, Duvall and Reneau 1962). Since samples contained proteins with high molecular weights application via the round window was not plausible as this is permeable to substances <500-1000 Da only (Juhn, Hamaguchi and Goycoolea 1989).

2.2. The Cochlear Perfusion Model

The cochlear perfusion method was used to assess the ototoxicity of all samples (method discussed in Nuttal, LaRouere and Lawrence, 1982). The method described here includes a number of modifications on the original method (Comis and Leng, 1979). Firstly, to reduce the increased longitudinal flow of cochlear fluid (> 600-fold) resulting from perforation of the cochlea (Ohyama, Salt and Thalmann *et al.*, 1988), the atlanto-occipital membrane covering cisterna magna was punctured. This enabled a study of the cochlear response to perfusates over a considerable period of time, whereas the entire perilymph would have been replaced in under 8 min if puncture of the atlanto-occipital membrane was not carried out (Ohyama *et al.* 1988). Secondly, since bacterial extracts and fractions are not produced in large quantities the method of perfusion of samples into the cochlea was modified in order to enable perfusion of small samples. Previously a microinfusion pump was used which requires a minimum volume of 1ml of test samples due to the large volume of dead space. Successful perfusions were achieved with volumes of test substances approaching 200µl using a 250µl fixed volume micropipette controlled using a hand perfusion kit.

2.2.1. Animal Selection and Exclusions

The animals used in the present study were pigmented adult guinea pigs weighing between 500 and 750g. Hearing was assessed superficially by selecting only animals with a positive bilateral Preyers' reflex. Statistical and summary data were excluded from animals with initial Compound Action Potentials (CAP) 'thresholds' (defined in next section) above a defined level as these animals are likely to have had pre-existing hearing loss. The defined levels were >50 dB SPL at 10 and 5 kHz, and 65dB SPL at 3kHz.

Anaesthesia was induced with an intra-peritoneal injection of 25% urethane (5.5ml/kg). Intra-peritoneal injections of 0.5 ml 25% urethane were administered to top up the anaesthesia if necessary. The animals were tracheotomised and the trachea was cleaned using tissue wicks at a minimum of half hourly intervals in order to maintain a patent airway throughout the experiment. The left auditory bulla was exposed via a ventrolateral approach involving removal of the posterior process of the inferior margin of the left mandible. A midline incision was made over the anteroposterior region of the skull and the temporalis and occipitalis muscles were reflected. The outer cartilaginous segments of the external auditory meati were carefully cut close to the skull to expose the meati without interrupting its patency (e.g. with leaked blood or flaps of cartilage). Puncture of the atlanto-occipital membrane covering cisterna magna was carried out to reduce longitudinal flow of the cochlea fluids (as discussed earlier in this chapter). The head of the animal was fixed within a stereotaxic frame using a jaw bar and 2 ear bars inserted into the external auditory meati. The temperature of the animal was monitored by an anal probe and maintained at 38°C using a thermostatically regulated heating table.

Once securely fixed within the stereotaxic frame the lateral aspect of the left bulla was fully exposed beneath the styloid and digrastic muscles using blunt dissection and retraction. The styloid process was removed to allow access for the micropipette (used for perfusions and recordings). The lower margin of the bulla was opened in order to expose the cochlea whilst maintaining the integrity of the tympanic membrane and the middle ear. A round hole of approximately 50μm diameter was bored into scala tympani at the basal turn of the cochlea (approximately 2mm from the round window) using a hand-held drill. A second, slightly larger hole was drilled at the apex of the cochlea to allow efflux of the perfusate. Any fluid that

accumulated around the cochlea as a result of cochlear fluid leakage or efflux during perfusion was mopped up using tissue wicks prior to all electrophysiological recordings. Auditory stimulation was provided by a piezoelectric transducer coupled to a hollow left ear bar. The auditory evoked potentials were measured before perfusion of test substances (control values), and then at 1 min, 5 min, 10 min and at 10 min intervals until 90 min post perfusion.

A summary diagram of the experimental set up can be seen in **Fig. 2.**

2.2.2 Perfusion of Samples into Scala Tympani

Test substances were administered using a glass micropipette connected via polythene tubing to a fixed volume Gilson pipette (250µl Micro/Pettor) connected to a hand perfusion kit. A stainless steel wire electrode was sealed within the micropipette for recordings of sound evoked potentials. The tip of the pipette (just less than 50 µm diameter) was inserted into the basal hole with a Bonetti micromanipulator to create a tight fluid seal. The test substance was perfused at a rate of approximately 10µl/min for one minute. Test substances were kept at room temperature (approximately 20°C). It is well established that the sound evoked potentials of the guinea pig cochlea are highly dependent on temperature (Inamura, Kusakari and Takasaka 1987). However, because exposure of the cochlea results in a significant drop in the temperature of the cochlea (to around 32°C) (Nuttall and LaRouere 1980), perfusion of the samples at room temperature reduces the temperature of the cochlea by only 1 or 2°C, and therefore produces little or no effect on the auditory potentials (Nuttall *et al.* 1982). The cochlea is capable of stable performance at this temperature (Nuttall *et al.* 1982), and any losses which are produced by a temperature drop are transient and thus distinguishable from sustained ototoxic events.

Where more than one substance was to be perfused into the cochlea, the following protocol was followed. After perfusion of the first substance and the completion of the required electrophysiological measurements, the micropipette was withdrawn from the cochlea, emptied and refilled with the second substance. The micropipette was then replaced and a series of control readings taken prior to the second perfusion. The time between perfusions varied according to the effect of the initial perfusion. In all cases, the second perfusion was not carried out until CAP

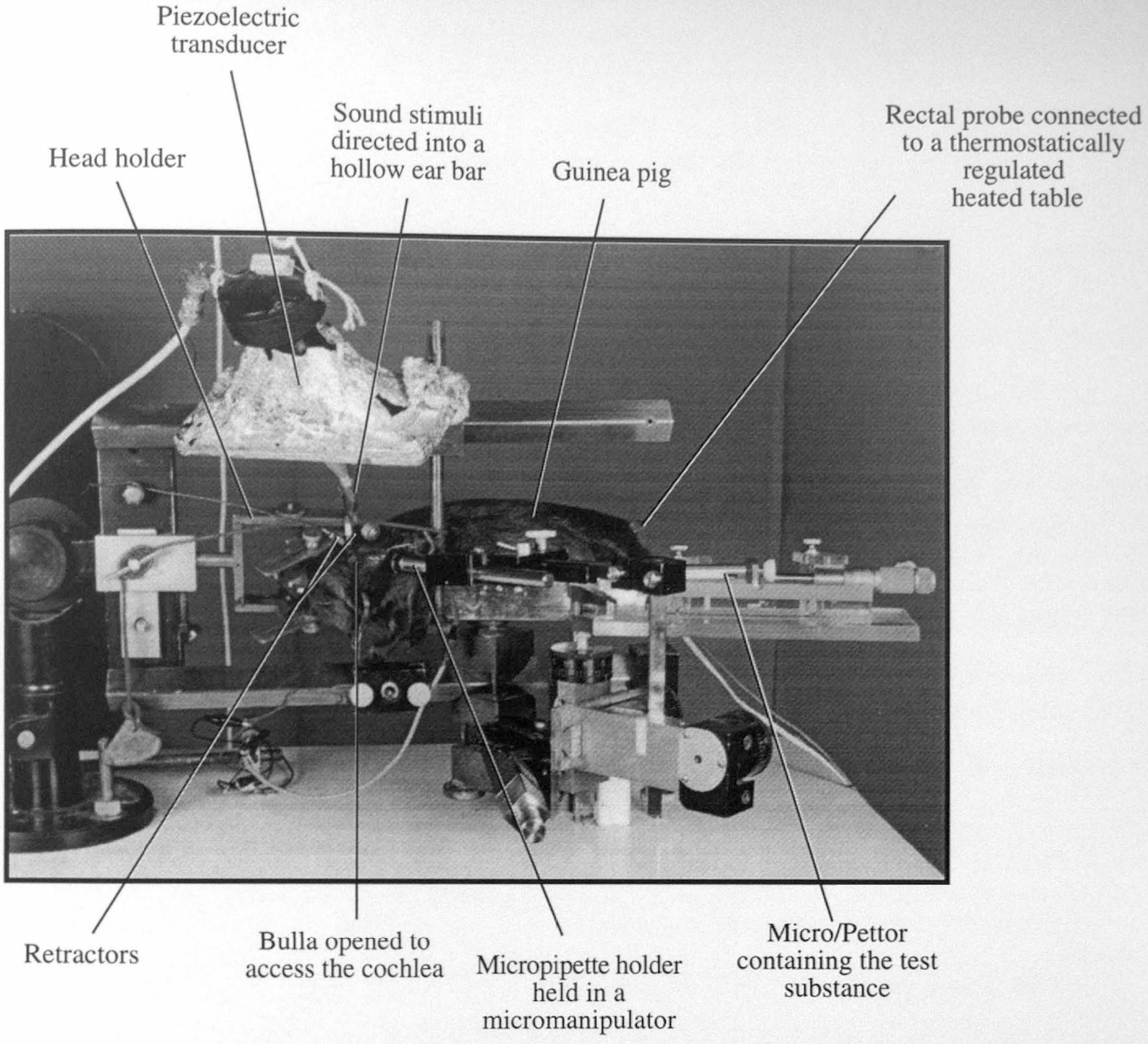


Figure 2. Labelled photograph to show the set up of the animal and equipment during recordings.

The Guinea pig is held in a head holder on top of a thermostatically regulated heated plate. The left bulla is opened to expose the cochlea into which a dual purpose micropipette/electrode is inserted. The pippete/electrode is connected to both the recording apparatus, and a Micro/Pettor containing the test substance. Sound stimuli are provided by a piezoelectric transducer which directs sound into the left ear via a hollow ear bar.

recording were stable (i.e. 2 or more recordings in which there was no more than 5dB difference in CAP recordings).

2.2.3 Fixation of Cochlear Tissue

Immediately after the final electrophysiological measurements, the left (treated) cochlea was fixed by intravital microperfusion of 100µl fixative (4°C) (2.5% glutaraldehyde in 0.05M BES ((2 hydroxyethyl)-2 amino-ethane sulphonic acid) buffer and 0.05M sucrose, **see appendix I**). Animals were then killed by transection of the brainstem, and both bullae were removed. The right (untreated) cochlea was exposed and fixed by removal of the very apical end of the cochlea and perfusion of the same fixative through the round window using the hand perfusion kit. Both treated and untreated cochleae were immersed in fixative and stored at 4°C for at least 24 hours.

2.2.4. Electrophysiological Recordings

The auditory evoked potentials measured during this study were the compound action potential (CAP) and cochlear microphonics (CMs).

Tone pips were generated by a gated oscillator (Farnell type LFM4). The opening time of the gate was phase locked to the incoming signal so that all tone pips started at point zero of the wave form. After attenuation the signal was used to drive the piezoelectric speaker. The intensity of the tone pips was calibrated using a B&K amplifier (type 2606) and microphone (type 4134). 0dB attenuation corresponded to 95, 90 and 105 dB SPL at 10, 5 and 3 kHz respectively. The cochlear responses were amplified (x 20k), filtered (300Hz-15kHz) and displayed on a MacLab.

CAPs were elicited by stimulation with a 10ms duration tone pip. The attenuation necessary to evoke a CAP of N1-P1 amplitude of 100µV (2V post amplification) was determined and defined as the threshold CAP for this study. The threshold was determined by averaging the responses to 8 tone pips (performed by MacLab) and alteration of the stimulus intensity until an average N1-P1 amplitude of 100µV was produced. A fall in the CAP was calculated as the increased intensity in sound (dB) required to restore the amplitude to 100µV. CAP thresholds were measured at 10, 5

and either 1 or 3 kHz (1kHz was measured in initial experiments, but 3kHz was adopted in later experiments due low sound pressure level produced by the equipment at this frequency).

CMs were elicited using tone pips of 10ms duration at 5kHz. The peak to peak amplitude of the CM traces produced in response to four pips were recorded and averaged (by a Gould (DSO) 1602 digital oscilloscope) over a range of stimulus intensities (-60 to 0 dB SPL in 10 dB increments).

2.2.5. Morphological examinations

It was not possible to carry out morphological examinations on all treated cochleae due to both financial and time constraints. Cochleae could be examined by either Scanning Electron Microscopy (SEM) or Transmission Electron Microscopy (TEM), but not both because specimen preparations were different for each. Specimen preparations were far simpler (and therefore cheaper and quicker) for SEM than TEM, and SEM allowed examination of the whole organ of Corti compared with small sections of the organ of Corti examined when carrying out TEM examinations. Therefore SEM examination was carried out on at least one cochlea from each treatment group which produced ototoxicity as judged by CAP and CM losses, whereas TEM examinations were carried out only when there were sufficient specimens and time available.

SEM

24hrs to 7 days post fixation modioli of both fixed treated and untreated cochleae were dissected out and the height of the cochlea reduced by removal of some of the apical end (to allow mounting into the electron microscopy specimen holders). 7 - 14 days post fixation modioli were serially dehydrated in acetone (1h in 50%, 70%, 80%, 90%, 95%, 100 %, and 100% acetone), and dried by the critical point drying method using liquid carbon dioxide. The modioli were then mounted on copper SEM holders using Araldite, and sputter coated with platinum (EMsope 5C500 low voltage sputter coater) to a depth of 12-15 nm as judged by the thickness monitor. Preparations were examined under a JEOL 120 CXII transmission electron microscope fitted with a TEMSCAN scanning attachment. The images were viewed by a secondary electron detector with an acceleration voltage of 40 kV.

TEM

12-48hrs post fixation modioli of treated cochleae (and some but not all untreated cochleae) were dissected out, and then treated with osmium tetraoxide for 30 min (1%(w/v) **see appendix I**). Modioli were then immersed in 70% ethanol and one or two large pieces (≈ half a turn) of organ of Corti dissected free. A basal portion of the organ of Corti was always removed, and an additional middle piece was taken from some cochleae (3 and 2 turns from the apex respectively). The samples were serially dehydrated in ethanol (15min each in 70%, 90%, 100%, 100%), transferred into propylene oxide (2x 15 min), and then into a 1:1 (v/v) solution of resin/propylene oxide for 1h. The samples were then embedded in degassed resin (a modification of Epon 812 see **appendix I**), and polymerisation was carried out over 24-48h at 60°C. An LKB Ultracut E ultramicrotome was fitted with a glass knife for cutting semithin sections, and with a diamond knife for cutting ultrathin sections. Semithin sections were stained with 1% toluidine blue and examined using a light microscope. Ultrathin sections were picked up on Formvar coated copper grids, stained with aqueous uranyl acetate and lead citrate in a Reichert Ultrostainer and examined using a JEOL 120CXII TEM with an acceleration voltage of 80 kV.

2.3. Perfusates

2.3.1. Dissolution and Dialysis

Prior to perfusion, all samples were either dissolved in or dialysed into artificial perilymph (APL) (see **appendix I**). Samples were dialysed at 4°C in a volume of APL equal to 1000 fold the sample size with at least two changes after a minimum of 3h each. Bacterial extracts were stored frozen in aliquots at -20°C and thawed just prior to perfusion. Dissolution of drugs and final dilutions of bacterial extracts were made with filter sterilised APL and were carried out immediately prior to perfusions.

2.3.2. Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma Chemicals Co. Ltd. Chemicals used for Part II of this study (Chapter 10) were Quisqualate (QA), Kainate (KA), Nitro-L-arginine methyl ester (L-NAME), Methyl-L-arginine (L-MA), Superoxide Dismutase (SOD), and Deferoxamine mesylate (DEF). All chemicals were of the highest purity commercially available.

2.3.3. Acquisition of Bacterial Extracts

All culture, harvesting and disruption of bacteria were carried out off site. The **pneumococcal extracts** were a generous gift from Prof. T Mitchell at Leicester (now Glasgow) University. Bacterial extracts were from the genetically mapped wild type isolate *S. pneumoniae* type 3 GB050Z, and the pneumolysin deficient mutant *S. pneumoniae* type 3 GB050Z which was constructed by insertion-duplication mutagenesis (see **appendix II**). Bacterial cells were harvested from the cultures in which they had grown by centrifugation (20 min, 8000 rpm, Sorvall GSA rotor), and resuspended in 60 ml phosphate-buffered saline. The bacterial cells were disrupted by sonication (Braun sonicator setting 70 for 6 x 30sec with 1 min cooling between bursts). The disruptates were clarified by centrifugation (30 min, 20,000rpm Sorvall SS34 rotor), filter sterilised (0.45mm acrodisc) and stored at -20°C until used. **Hib and meningococcal extracts** were purchased from Centre for Applied Microbiology and Research (CAMR) at Porton Down, Wiltshire. Hib and meningococcal extracts were from clinically isolated strains (a group B meningococcal isolate received from Prof. John Heckels at Southampton Univeristy, and Hib St Chauhan strain). The **Hib bacterial extract** was prepared as follows. Bacterial plates were grown at 37°C in 5% CO_2, liquid cultures at 37°C, shaken at 150rpm, and fermented at for 41h. Bacterial cells were separated from the culture in which they had been grown by filtration. The cells were then disrupted chemically and physically. Chemically the cells were lysed by mixture with EDTA (70ml of 10mM EDTA at pH 7.0), and physically the cells were disrupted by two passes through a French pressure cell (compression force of 20,000 psi). The resultant cell disruptate was centrifuged at 16,000 rpm for 20 minutes at 4°C. The supernatant was removed and freeze dried in 10ml aliquots. The **meningococcal bacterial extract** was prepared using the following method. Bacteria were grown on brain heart infusion (BHI) slopes (+ NAD and haemin) at 37°C for 48h. The culture was washed from each of fours slopes (using 2ml medium) and used to innoculate 20ml BHI (+ supplements) at 37°C for 24h (250 rpm). This 20ml was then used to innoculate

200ml BHI (+ supplements) at 37°C for 16h (200 rpm).This 200ml was then used to innoculate a 5 litre fermenter at 37 for 6h (2.5 litre/min 500rpm stirring). Bacterial cells were harvested from a 5 litre fermentation by filtration (0.45u-0.2u) and resuspended in PBS (83ml). After storage at –20°C, cells were disrupted by two passages through a French pressure cell (compression force of 13 000 psi). The disrupted material was then centrifuged (20 000 rpm, 90 min), the top layer of the supernatant was removed (15ml) and the remaining supernatant was made up to 1 litre with PBS, filtered, and freeze dried in 50 ml amounts.

2.3.4. Treatments of Bacterial Extracts

Part I of this study was carried out in collaboration with Dr Lynn Dover who was responsible for all treatments of bacterial extracts which were carried out at the University of Birmingham.

2.3.4.1. Reconstitution of bacterial extracts

To each freeze dried aliquot of Hib bacterial disruptate 8ml of endotoxin free water was added and the material was allowed to dissolve on ice for 20 minutes. The volume of the solution was measured and adjusted to 10ml with endotoxin free water. Large particles of insoluble material were removed by centrifugation at 3300xg at 4°C for 10 min. The supernatant fluid was withdrawn and sterilised by filtration through Minisart filter (pore diameter = 0.22μm; Sartorius) into a sterile universal bottle. In this state the extract was known as a crude extract. The crude extract was then dispensed into 1ml aliquots and stored at -20°C until used for bioassay or further fractionation.

2.3.4.2. Sterility testing

Upon arrival at Birmingham University, all bacterial extracts were tested for sterility prior to any perfusions. In addition, all samples were filter sterilised through a 0.22μm filter following reconstitution.

2.3.4.3. Concentration of bacterial extracts

Bacterial extracts were concentrated by two methods. Initially extracts were concentrated using Sephadex G-10 which absorbs molecules of molecular mass below the exclusion limit of the Sephadex (1500 Da for globular proteins), i.e. large solutes are concentrated without simultaneous

concentration of buffer components. Sephadex was added to bacterial extracts, and the swollen sephadex was removed by centrifugation. Later in the study, Centriplus 10 filters (Amicon; Nominal molecular weight cut off of + 10kDa) were used instead of Sephadex G-10 to reduce any loss of extract proteins which may have occurred by adsorption to the concentrating medium.

2.3.4.4. Thiol activation of bacterial extracts and bovine serum albumin (BSA)

The reducing agent Dithriothreitol (DTT) was added to each extract to a concentration of 1mM and the extracts were then held on ice for 3 hours followed by dialysis in degassed APL in full sealed bottles to remove the reducing agent and limit the re-oxidation of the sample. The bicarbonate component of APL was added after degassing to prevent pH alterations caused by CO_2 removal. In order to assess the non specific effects of thiol activation on the cochlea, DTT treated bovine serum albumin (BSA) was tested for ototoxicity. BSA was chosen as it contains numerous disulphide bonds (Swissprot). 2mg/ml BSA (equal concentration to bacterial extracts) was DTT treated exactly as described for bacterial extracts.

2.3.4.5. Ultracentrifugation of bacterial extracts

Extracts were centrifuged at 500,000 x g at 4°C for 16 h initially, and for 30min in all further ultracentrifugations. The supernatant fluid was carefully withdrawn and stored in aliquots at -20°C. The deposited material was gently resuspended to the original volume in APL using a glass rod. After transfer to Eppendorf tubes the material was sonicated for 30 min in a cup horn sonicator containing iced-water. In some experiments, this pellet material was then centrifuged again to remove any contaminating cytoplasmic components before re-suspension in APL by sonication. This material was termed "washed pellet".

2.3.4.6. Fractionation of the supernatant by Anion exchange chromatography

All fractions were carried out using Fast flow - diethylaminoethyl (DEAE) sepharose. Small columns were poured (Bed volume = 1.5ml) and equilibrated by washing with 50ml 50mM Tris.HCl, pH7.5 overnight. Samples containing 15mg protein (volume @ 1ml) were loaded onto the columns and non-adsorbed proteins were eluted using 20ml 50mM Tris.HCl pH7.5. Adsorbed proteins were eluted using various concentrations of sodium chloride in the above buffer. Step elution schemes were carried out using eluents in 10ml aliquots. Eluates were all collected and re-concentrated to <1ml and were then dialysed overnight. Eluates were diluted with enough sterile

APL to return the samples to their original volumes.

In order to establish the elution characteristics of the extracts from the DEAE-seahorse, an **elution profile** was produced by using the fractionation procedure as described above, except that the Tris eluted sample was collected in 9 fractions (600–1200µl each) and the 1M NaCl eluted sample was collected in 6 small fractions (600–1300µl each). Protein determinations were carried out for all fractions.

2.3.4.7. Bulking of bacterial extract fractions with Bovine Serum Albumin (BSA)

Fractions of bacterial extracts were bulked with BSA to reduce any digestion of ototoxins by co-purification with protease/s. Two schemes were used, fractions were either supplemented with BSA to 10 $mg.ml^{-1}$ immediately after elution or were eluted with eluents containing 10 $mg.ml^{-1}$ BSA.

2.3.4.8. Adsorption Of Pneumolysin From Wild Type Cell Extracts

Immunopurification of wild type pneumococcal extract was carried out by Prof. Mitchell at Leicester University. An immuno-affinity chromatography column was made by coupling rabbit anti-pneumolysin immunoglobulin G to a Hi-Trap NHS activated column following the manufacturers' instructions. The haemolytic titre of the wild type extract was determined by dilutions in PBS followed by the addition of 2% sheep erythrocytes. The endpoint was taken as the well in which 50% of the cells had lysed after 30 min at 37°C. 5ml extract from the wild type pneumococcus GB050Z was applied slowly to the anti-pneumolysin affinity column followed by 5ml PBS. The column eluate was passed through the column a further three times. This adsorbed extract was found to have no detectable haemolytic activity and was thus considered to be free of pneumolysin. The sample was stored at -20°C until used.

2.3.5. Protein analysis of Bacterial Extracts

All protein analysis was carried out by Dr Lynn Dover.

2.3.5.1. Protein Content Determinations

The Biorad protein assay reagent was used to determine protein concentration in bacterial extracts. Slight modifications to the manufacturers' protocol were used. Samples of bacterial

extracts of an appropriate volume were taken and diluted with water to a final volume of 100ml. Each sample was then mixed with 150ml 0.2M NaOH in a disposable cuvette before the addition of 3ml Protein Assay reagent (Na OH was added to disrupt any protein-bearing membrane vesicles). After 2 minutes' incubation at room temperature the protein content of each sample was quantified by measuring A_{595nm}. Bovine serum albumin was used as a standard for all protein determinations.

2.3.5.2. Polyacrylamide gel electrophoresis of bacterial extracts (PAGE)

Sodium dodecyl sulphate (SDS)-PAGE

Standard protocols were used for the separation of proteins by SDS-PAGE. Homogeneous resolving gels (160 x 120 x 1mm) were cast at 12%T (w/v total monomers), 2.67%C(w/w cross linker, bis-acrylamide). Samples were prepared for electrophoresis by mixing with an equal loading buffer (62.5mM Tris.HCl, pH6.8, 0.02% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 20 % (v/v) glycerol, 0.025% (w/v) bromophenol blue) and were held at 100°C for 3 min. Electrophoresis was carried out at a constant current of 35mA for 2h at 4°C. Protein bands were visualised by staining with Coomassie Brilliant Blue G-250.

Native-PAGE

The electrophoretic conditions were the same as used for SDS-PAGE other than SDS was omitted from all buffers, resolving gels were cast at 5%T, 2.67%C and samples were prepared for electrophoresis by mixing with an equal volume of sample loading buffer which contained no β-mercaptoethanol and SDS. Samples were not heat denatured prior to electrophoresis. Protein bands were visualised by staining with Coomassie Brilliant Blue G-250.

2.3.5.3. Staining of gels with Coomassie Brilliant Blue

Gels were stained for 3h in a solution comprising 0.05% (w/v) Coomassie Brilliant Blue G-250 in glacial acetic acid/methanol/water (1:5:5 by vol) and were then destained overnight using 10% methanol (v/v), 7.5% (v/v) acetic acid in distilled water. Stained gels were dried and recorded by optical scanning with an Epson GT9000 scanner. The brightness and contrast of the scanned images were adjusted to achieve an accurate representation of the stained gel using Adobe Photoshop 3.0.

2.3.5.4. Detection of pneumolysin in bacterial extracts by Western Blot

After electrophoresis of bacterial extracts on either SDS- or native-PAGE, proteins were transferred to nitro-cellulose filters (BioTrace NT, Gelman Sciences Inc., 0.45mm pore size) overnight at a constant current of 0.1mA at 4°C in 0.3% Tris, 1.44% glycine, 0.1% SDS. All washes and incubations of filters were carried out at 25°C in hybridisation tubes within a Hybaid hybridisation oven. The nitro-cellulose filters were saturated with protein to reduce non-specific antibody binding by incubating with 50ml 2% blocking solution (Boehringer Mannheim) for 2h. A 1θ° antibody solution was prepared as a 1:1000 dilution of MAb7 stock (anti-pneumolysin monoclonal antibody) in phosphate buffered saline (PBS). The blocking solution was removed and filters were incubated with 10ml of primary monoclonal antibody solution at 25°C for 2h. In order to remove excess Mab7, filters were then washed three times with 30ml PBST (0.0005% Tween 20/PBS) for 10min each. The filters were incubated with a detection conjugate (1:500 dilution Goat, anti-mouse IgG alkaline phosphatase conjugate in PBST) (Sigma Immunochemicals) for 2h. Excess detection conjugate was then removed by washing three times with 30ml PBST for 10min each. Conjugate-labelled immune complexes were visualised by incubation with the precipitating substrate system for alkaline phosphatase. BCIP/NBT (0.56mM 5-bromo-4-chloro-3-indolyl phosphate, 0.48mM nitro blue tetrazolium, 59.3mM $MgCl_2$ in 10mM Tris, pH 9.2) (Sigma Chemicals) for 20 min at room temperature in a plastic dish. The reaction was stopped by washing the filter in de-ionised water and the results recorded by optical scanning with an Epson GT9000 scanner. The brightness and contrast of the scanned images were adjusted to achieve an accurate representation of the stained gel using Adobe Photoshop 3.0.

CHAPTER 3

Results Introduction

3.1 Interpretation of Electrophysiological Data

Before describing the experiments in this section, it is important to restate the principles used to govern the interpretation of the data. Experimental data produced by the cochlear perfusion model shows a moderate degree of variability. The degree of variability is dependent on the ototoxic potency of the test substance, and also on the physiological condition of the animal which can deteriorate. In addition, the guinea pigs used were not inbred and showed some inter-animal variability in their susceptibility to ototoxic factors. However, if interpreted with caution this model is a good one to detect ototoxicity.

Control experiments were carried out involving the perfusion of artificial perilymph (APL) into scala tympani and the assessment of degree of hearing loss. Typically, control perfusions produced a loss in the CAP of between 0 and 8 dB and loss in the CM between 0 and 25% at 90 min post perfusion; however in some cases the CAP and CM did decline to 15dB and 30% losses respectively. Data from within experimental programmes can also act as sufficient, if not superior controls to those of APL perfusions, and also reduce the number of animals used. Based on evidence from APL controls, and data from within experimental programmes, I have defined CAP and CM losses as 'positive' if they are $\geq$20dB or $\geq$40% respectively. These are arbitrary cut off levels, above which I am confident that losses indicate a genuine ototoxic effect. Losses below these levels may be indicative of ototoxicity - particularly losses in CAPs of 15-20dB, and losses in CMs of 30-40%. However, losses in this range will be interpreted individually in the light of other pertinent data when attempting to assign biological significance. I have also defined ototoxicity as *potent* and *moderate* if CAP losses observed were >40dB and between 20 and 40dB respectively.

Sometimes, an increase in all auditory potentials was seen immediately following perfusion. This may have been due to poor initial coupling of the fluid bathing the recording electrode with the perilymph of the cochlea prior to perfusion. In such cases, hearing losses were recalculated by taking the 1min recordings as the control values.

The initial auditory thresholds of all data presented in this thesis can be seen in **Fig. 3**. The thresholds varied according to frequency and showed a considerable range which was a result of both animal variability, and positioning of the basal hole. Within this study I have defined a threshold value (50dB SPL at 10 or 5kHz, and 65dB SPL at 3kHz), above which animals are likely to have had pre-existing cochlear damage. Within each chapter, I have also identified any animals which were clearly presenting auditory thresholds which were well outside the normal distribution of that group. Data will be presented from animals which displayed initial CAP thresholds above the defined level, or well above the normal distribution of the group, however the data has been excluded from statistical analysis and summary charts. From my experience with this model, animals which present high initial auditory thresholds often demonstrate a reduced sensitivity to ototoxic stimuli in comparison to animals with lower initial thresholds, however in the majority of cases, these animals will still display some sensitivity to potent ototoxic agents (as judged by agents which produce >40dB CAP loss in animals with initial thresholds within the normal distribution). Therefore I believe that data from these animals can contribute valuable information if interpreted with caution.

The CAP could be accurately determined to within ±1dB in the majority of cases. Occasionally an individual animal showed greater variability, but by increasing the number of CAPs averaged, measurements of the CAP were accurate to at least ± 3dB. In early experiments the CAP was recorded at 10, 5 and 1kHz. It often proved difficult to elicit a CAP with a 1kHz stimulus therefore the 1kHz CAP was replaced with the 3kHz CAP. The synchronicity of action potentials in individual fibres reduces as you move from the base to the apex of the cochlea. As substantial synchronicity is necessary to achieve a CAP, it becomes increasingly difficult to elicit a CAP with stimuli of decreasing frequency. At 3kHz it was sometimes difficult to elicit a CAP due to this

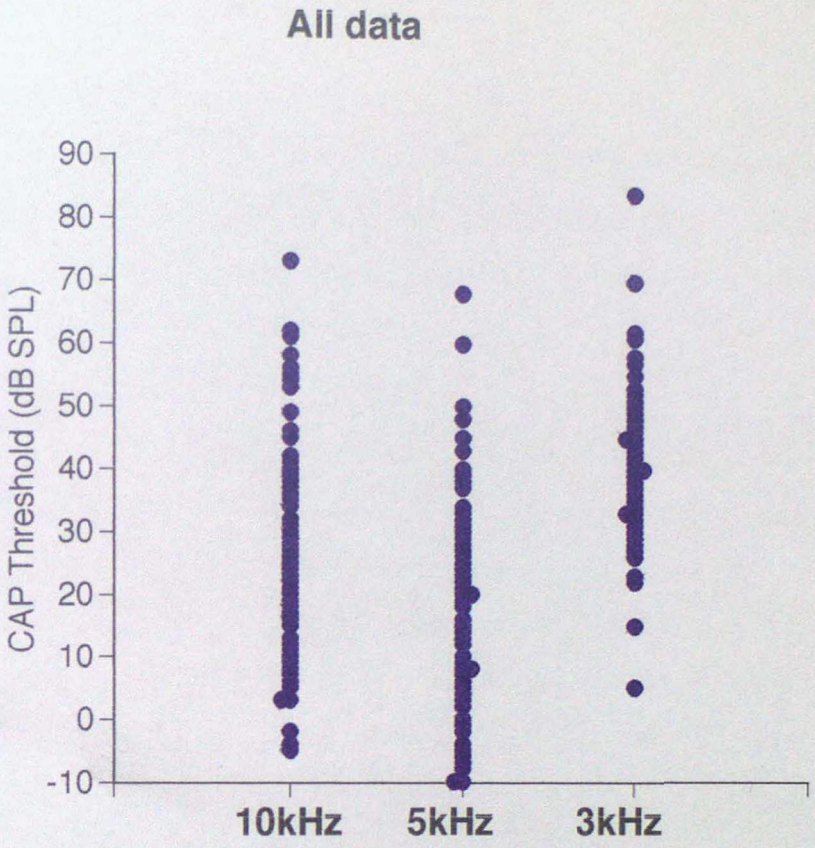


Figure 3 Threshold CAP values of all animals used in the work described in chapters 4-9.

problem; however the 3kHz CAP was considerably more reliable than the 1kHz CAP. It was sometimes difficult to measure a 5kHz CAP because of interference by the cochlear microphonic which masked the underlying CAP, and could not always be filtered out. For the above reasons results of the 5, 3, and 1 kHz CAP are not included in all the data sets.

As previously discussed (**Section 2.2.2.**), hypothermia of the cochlea produces a fall in auditory potentials. In some experiments a transient loss in the CAP was seen immediately post perfusion. This was seen most commonly at the 10kHz frequency. Although every attempt was made to ensure that each sample was perfused into the cochlea at room temperature, some samples (retrieved from the ice box in which they were held before use) may have been insufficiently equilibrated to ambient temperature when perfused. Therefore it is likely that perfusion of samples below ambient temperature into the cochlea was responsible for at least some of the transient losses in the CAP seen immediately post perfusion. The pronounced loss in the CAP at 10kHz may have been due to a basal-apical difference in response to cooling as described by (Ohlemiller and Siegel 1992), but it may also reflect the timing of the response as the 10kHz CAP was the first recording measured after perfusion.

3.2 Presentation Of Experimental Data And Statistical Analysis

A loss in CAPs was measured as the increase in stimulus intensity necessary to restore the N1-P1 amplitude to its reference value of 100μV. A loss in CM was calculated as the percentage drop in the peak CM with respect to the peak control value (i.e. 100-(X/control x100)), and rounded to the nearest whole figure. A decrease in the size of CAP or CM is expressed as a positive loss, and an increase is expressed as a negative loss. Positive ototoxicity has been defined as a loss in the CAP in excess of 20dB, and a loss in the CM in excess of 40%. Potent ototoxicity has been defined as a loss in the CAP in excess of 40dB. Average losses are expressed as the mean ± standard deviation. Where standard deviations are not expressed the result is from one animal only. The data are presented in two forms. All original data are presented graphically to allow rapid assimilation of patterns of change. They are also presented in tabular form from which mean losses and standard deviations are calculated in order to compare data between groups. Within

each group the number of animals used varied. This was necessary in order to keep the number of animals to a minimum.

Statistical analysis was carried out on 10kHz CAP losses at all time points between 1 and 90 min post perfusion. The CAP was analysed at 10kHz as this is the most sensitive recording and thus is most likely to reveal differences between treatment groups. Repeated measures ANOVA was carried out between all treatment groups within each section to determine the statistical significance of treatment at all time points, and also to determine the statistical significance of the change in the CAP with time.

CHAPTER 4

Ototoxicity In Haemophilus influenzae Type b (Hib) Bacterial Disruptate

It had been previously demonstrated that a potent ototoxic factor exists in the crude cytoplasmic extracts of Hib. This potent ototoxic factor is inactivated by mild heating (56°C for 10 min) or by treatment with a proteinase (insoluble proteinase K complexed to acrylic beads) and is vastly more ototoxic than phenol/water extracts of LOS (Amaee 1995). Thus it appears that there may be a protein toxin in the cytoplasm of Hib which is potently ototoxic. In this section an attempt was made to purify and characterise the ototoxic factor. To my knowledge this is the first attempt to purify and characterise an ototoxic factor in the cytoplasmic contents of Hib.

Four batches of Hib were grown and worked up at CAMR. One batch was used for all the experiments described in this section. In total, 40 animals were used in this chapter, and the initial auditory thresholds can be seen in **Fig. 4**. Data from three animals were excluded from statistical and summary information as one displayed initial auditory thresholds above the defined level, the second animal died during the experiment, and the third was killed at 50 min.

Aims and Objectives

1. To fractionate the bacterial disruptate into a soluble fraction and pellet by ultracentrifugation.
2. To fractionate the soluble fraction by anion exchange chromatography.
3. To perfuse the crude and fractionated bacterial extracts into scala tympani and monitor electrophysiological responses.
4. To examine by electron microscopy perfused cochleae if ototoxicity was observed in terms of electrophysiological responses.

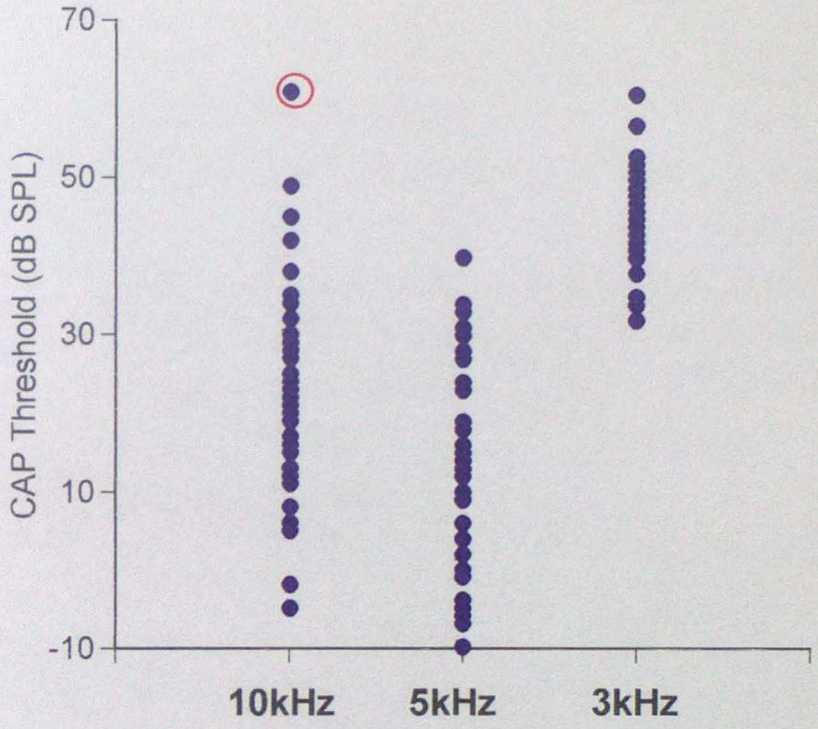


Figure 4. **Threshold CAP values of all animals used in the work described in this chapter.**

Red circles indicate thresholds either above the defined level (50dB at 10 and 5kHz and 65dB at 3kHz), or well outside the normal distribution of the group.

4.1 Crude Hib Bacterial Disruptate

4.1.1 Undiluted Crude Disruptate

The crude Hib bacterial disruptate was tested for ototoxicity in three animals **(Fig. 4.1.1; Table 4.1.1)** and produced positive 10kHz CAPs losses in all animals. At 90 min, 10kHz CAP losses in animals 1, 2 and 3 were 45, 25 and 78dB respectively. Animal 3 displayed positive losses in CAPs at all frequencies within 5 min of perfusion. Animals 2 and 3 displayed no positive CM loss, whereas animal 1 exhibited a positive loss in the CM of 54% at 90 min.

Table 4.1.1: Electrophysiological losses evoked by perfusion with crude Hib bacterial disruptate in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 1				Animal 2				Animal 3			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	30	27	67	0.22	45	40	-	0.26	-5	-10	35	0.26
	Loss in CAPs (dB) or CM (% control)											
1	7	8	1	-1	9	6	-	-12	**55**	**38**	19	-12
5	**27**	**26**	3	34	13	12	-	-5	**75**	**48**	**21**	-5
10	**31**	18	3	6	19	14	-	21	**75**	**50**	**25**	-3
30	**36**	-	6	15	19	14	-	2	**75**	**51**	**28**	8
60	**40**	-	10	36	**27**	19	-	19	**77**	**55**	**29**	24
90	**45**	-	13	**54**	**25**	17	-	14	**78**	**54**	**31**	28

4.1.2 Dilution of the Crude Hib Bacterial Disruptate I (1:10 dilution)

For future reference the crude disruptate was diluted and assessed for ototoxicity in order to determine minimal concentrations which were active. Initially the sample was diluted to 1:10 and tested for ototoxicity in three animals **(Fig. 4.1.2; Table 4.1.2)**. Perfusion of crude Hib disruptate diluted 1:10 produced positive losses in CAPs in all animals. At 90 min, 10kHz CAP losses were 28, 45, and 23 dB in animals 4, 5 and 6 respectively. Positive CM losses were not displayed by any animal.

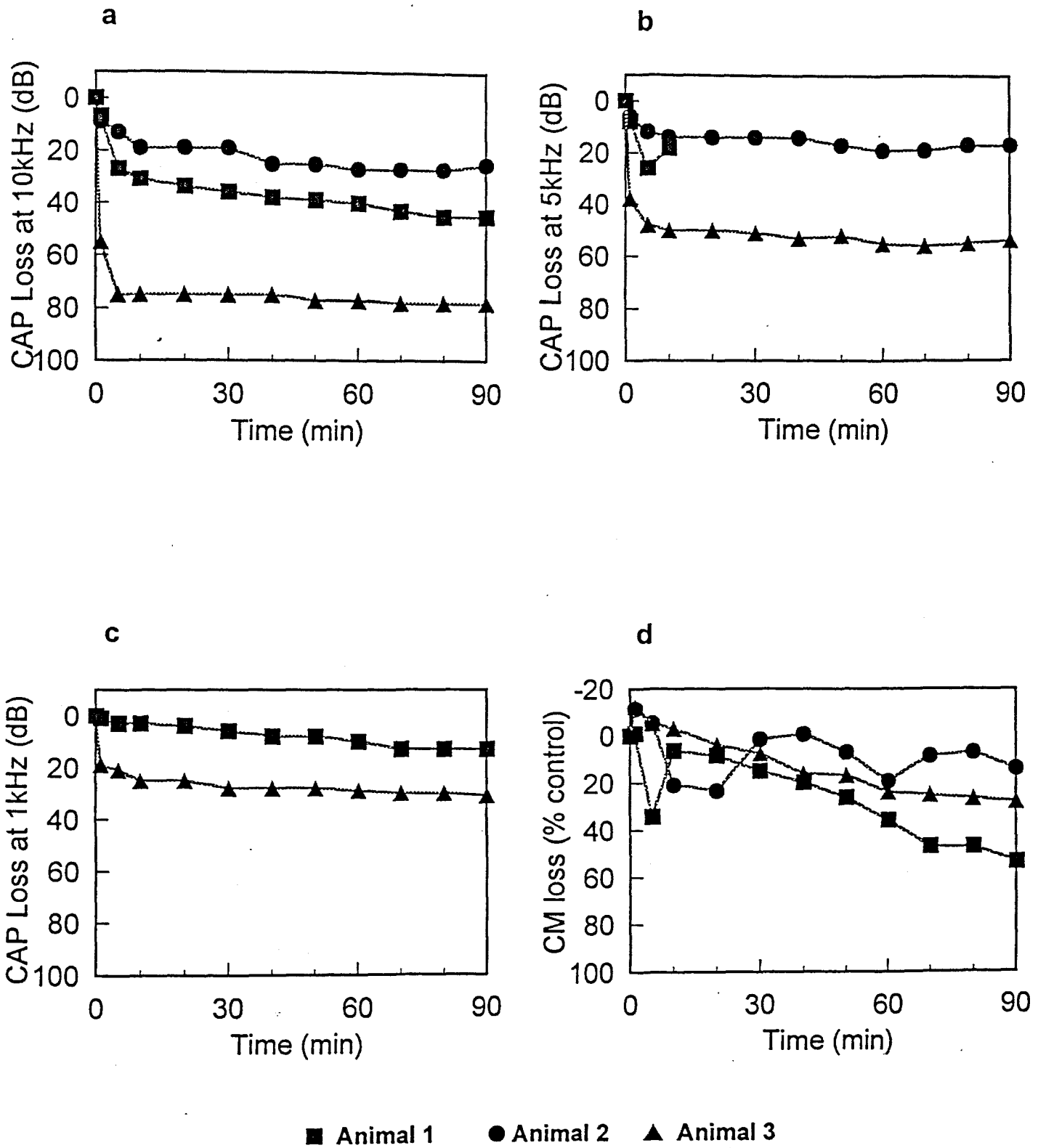


Figure 4.1.1. Losses in the CAP and CM in three animals, each treated with crude Hib bacterial disruptate.

a-c) Losses in the CAP elicited with 10, 5 and 1 kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

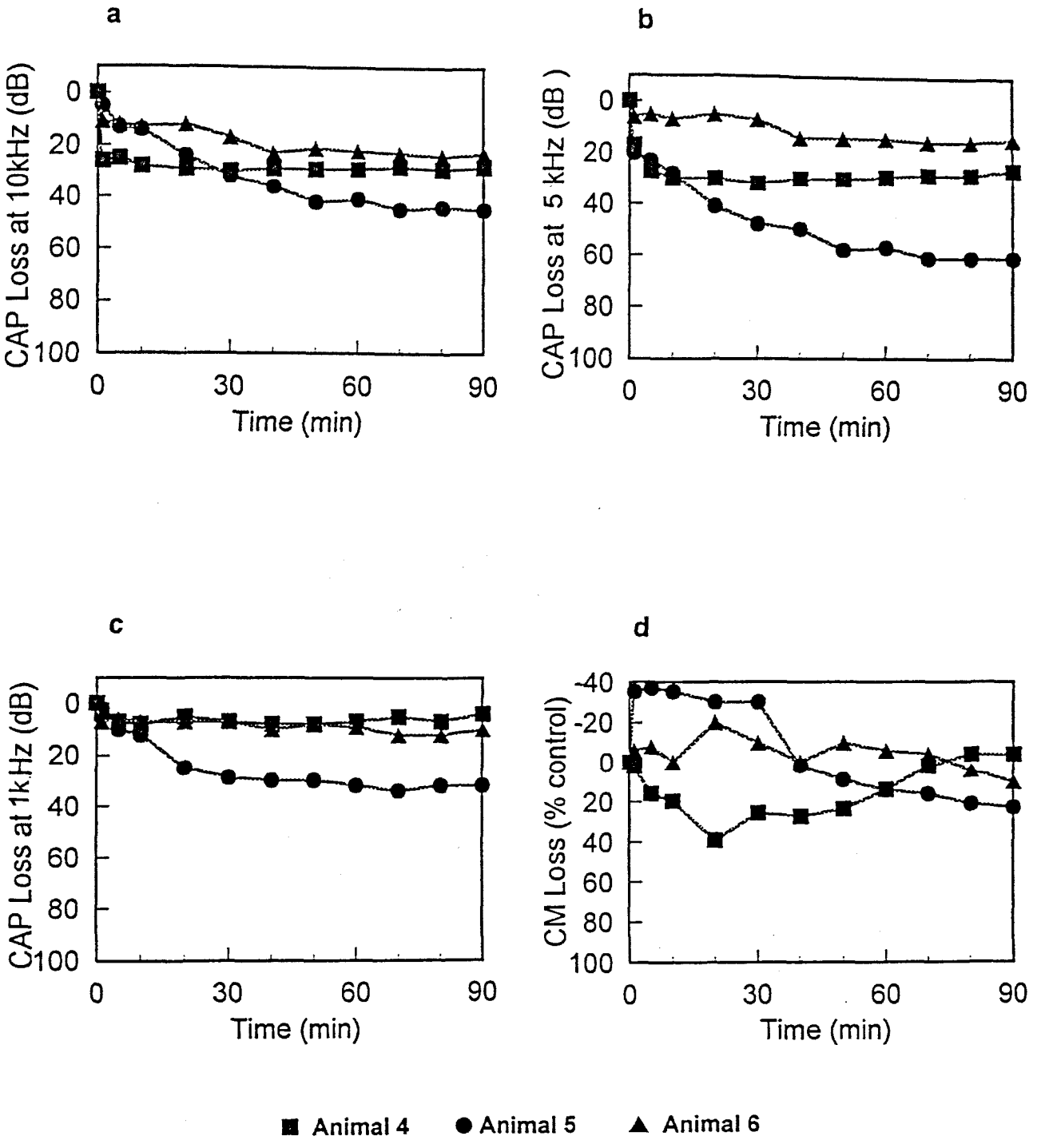


Figure 4.1.2. **Losses in the CAP and CM in three animals, each treated with 1:10 crude Hib bacterial disruptate.**

a-c) Losses in the CAP elicited with 10, 5 and 1kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

Table 4.1.2 : Electrophysiological losses evoked by perfusion with crude Hib bacterial disruptate diluted 1:10 in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 4				Animal 5				Animal 6			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	-5	-10	41	0.18	29	-5	41	0.20	32	23	43	0.18
	Loss in CAPs (dB) or CM (% control)											
1	**26**	17	4	2	5	**20**	2	-35	11	6	7	-6
5	**25**	**27**	7	16	13	**23**	10	-37	12	5	6	-8
10	**28**	**30**	8	20	14	**28**	12	-35	13	7	7	0
30	**30**	**32**	7	26	**32**	**48**	**29**	-30	17	7	7	-10
60	**29**	**29**	7	14	**41**	**57**	**32**	14	**22**	14	9	-6
90	**28**	**26**	4	-4	**45**	**61**	**32**	23	**23**	14	10	10

4.1.3 Dilution of the Crude Hib Bacterial Disruptate II (1:20)

As the initial dilution of Hib extract displayed potent ototoxicity, the crude Hib disruptate was further diluted to determine the threshold concentration of ototoxic activity. Crude Hib disruptate diluted 1:20 was tested for ototoxicity in three animals (**Fig. 4.1.3**; **Table 4.1.3**). Positive losses in CAPs were seen in animal 7 at 1 min which recovered by 5 min, indicating a temperature effect (discussed in section 3.1.). But, at 90 min animal 7 lost 28dB in the 10kHz CAP, and animal 8 lost 22dB in the 5kHz CAP. No positive loss in the CM was seen in either animal, however animal 7 displayed increases in the CM in excess of 60% at most time points.

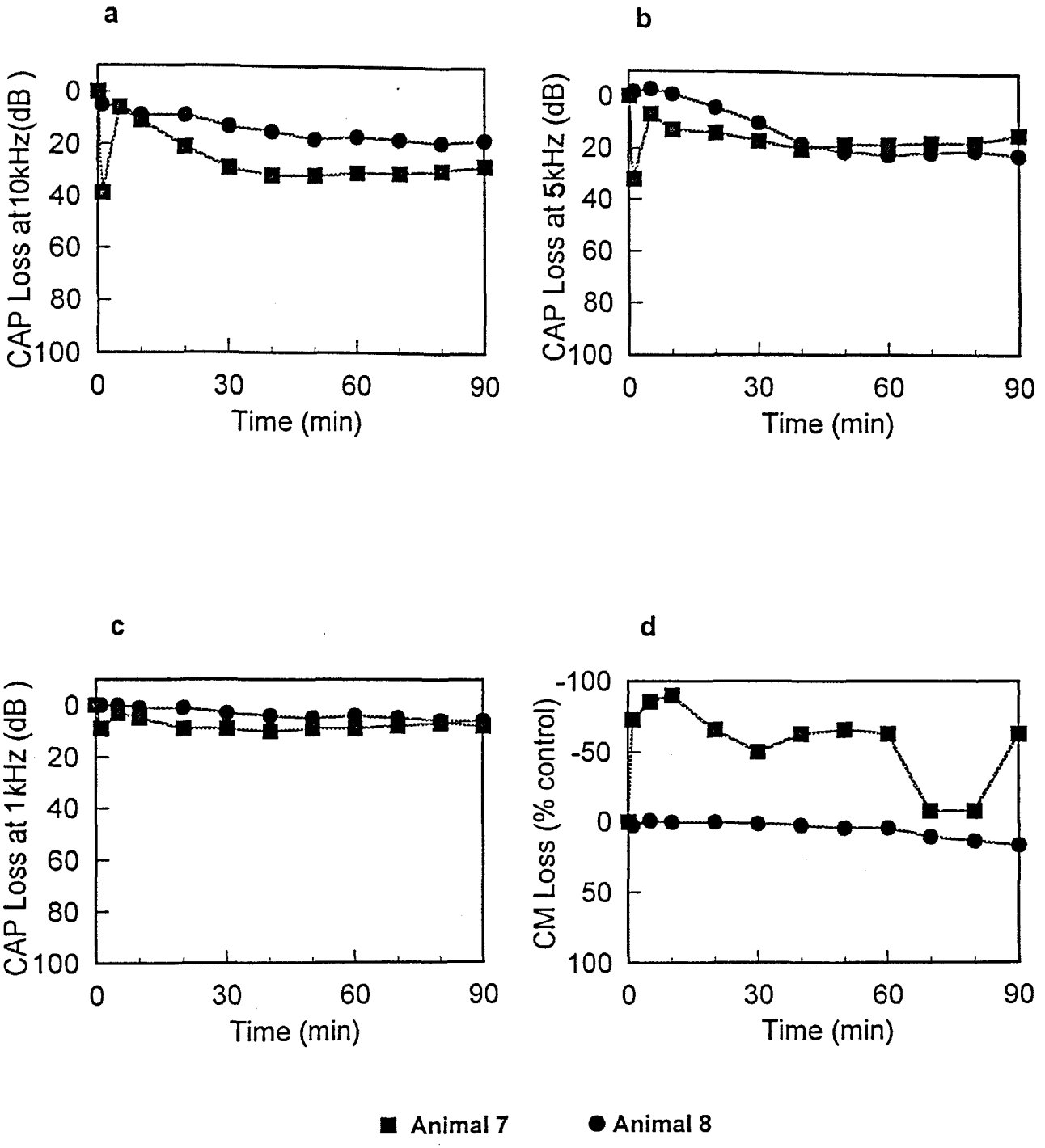


Figure 4.1.3. **Losses in the CAP and CM in two animals, each treated with 1:20 crude Hib bacterial disruptate.**

a-c) Losses in the CAP elicited with 10, 5 and 1kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

Table 4.1.3 : Electrophysiological losses evoked by perfusion with crude Hib bacterial disruptate diluted 1:20 in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 7				Animal 8			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	24	13	50	0.14	19	-6	42	0.34
	Loss in CAPs (dB) or CM (% control)							
1	**39**	**32**	9	**-73**	5	-2	0	2
5	6	7	3	**-86**	6	-3	0	-1
10	11	13	5	**-90**	9	-1	1	0
30	**29**	17	9	**-50**	13	10	3	1
60	**31**	18	9	**-63**	17	**22**	4	4
90	**28**	14	8	**-63**	18	**22**	6	16

4.1.4 Dilution of the Crude Hib Bacterial Disruptate III (1:50)

As the 1:20 dilution of the crude Hib bacterial disruptate displayed some ototoxicity, crude Hib bacterial disruptate was diluted to 1:50 and assessed for ototoxicity in one animal (**Fig. 4.1.4; Table 4.1.4**). Animal 9 has been excluded from statistical analysis as the animal was killed after the 40 min recording. Perfusion of crude Hib bacterial disruptate diluted 1:50 produced no positive losses in CAPs or CM at 40 min. Losses did not exceed 10dB and 7% in the CAPs and CM respectively.

Table 4.1.4 : Electrophysiological losses evoked by perfusion with crude Hib bacterial disruptate diluted 1:50 in one animal. Positive losses are bold.

Time post perfusion (min)	Animal 9			
	CAP			CM
	10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) Peak CM (mV)			
	11	6	47	0.34
	Losses in CAPs (dB) and CM (% control)			
1	-1	2	-3	0
5	2	3	-2	3
10	6	7	0	7
30	8	8	0	3
40	7	10	0	7

4.1.5. Crude Hib Bacterial Disruptate : Summary and Conclusions

Average 90 min losses in CAPs and CMs produced by crude Hib bacterial disruptate extracts are summarised in **Table 4.1.5**.

Table 4.1.5: Average 90 min losses evoked by perfusion with Crude Hib bacterial disruptate extracts. Positive losses are bold.

Treatment (Crude Hib Disruptate)	Average losses (± SD) in CAPs (dB) or CM (%) at 90 min post perfusion			
	CAP at 10 kHz	CAP at 5 kHz	CAP at 1kHz	CM
Undiluted (n=3)	**49 ± 27**	**35 ± 26**	**22 ± 13**	32 ± 20
Diluted 1:10 (n=3)	**32 ± 12**	**34 ± 24**	15 ± 15	10 ± 13
Diluted 1:20 (n=2)	**23 ± 7**	18 ± 6	7 ± 1	-23 ± 56

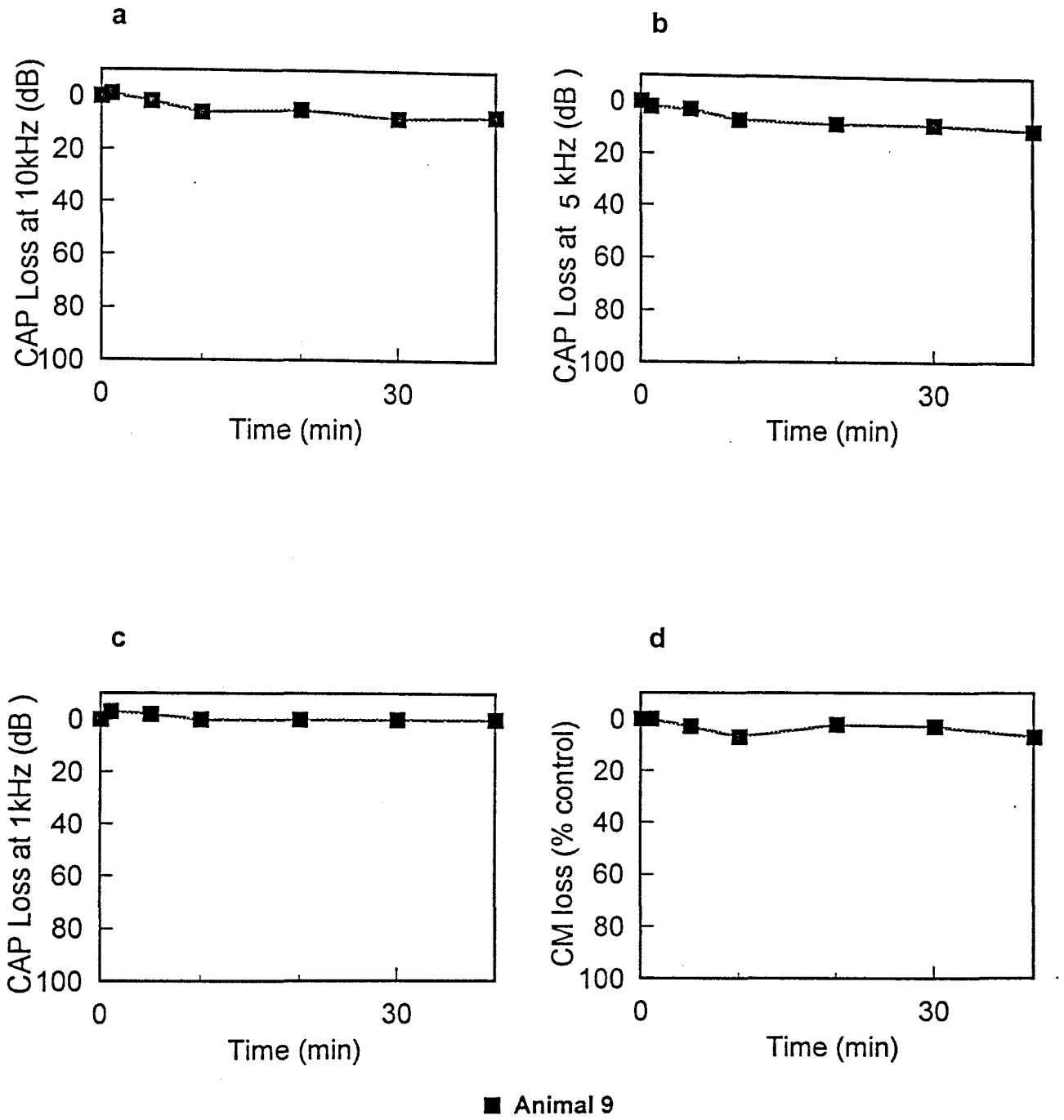


Figure 4.1.4. **Losses in the CAP and CM in one animal, treated with 1:50 crude Hib bacterial disruptate.**

a-c) Losses in the CAP elicited with 10, 5 and 1 kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

Conclusions

- There was evidence for **sustained** ototoxicity in undiluted, 1:10 and 1:20 crude Hib bacterial disruptate. There was no evidence for sustained ototoxicity in 1:50 crude Hib bacterial disruptate, however this animal was killed after the 40 min recording.
- There was also some evidence of **transient** ototoxicity in 1:20 crude Hib bacterial disruptate. However this was not consistent between animals and was likely to have been due to incomplete equilibration of the sample with ambient temperature.
- Therefore, ototoxicity activity in crude Hib bacterial disruptate is dose dependent, and threshold concentration for ototoxic activity (within 90 min) is >1:20 dilution.

4.2. Ultracentrifugation I (16h at 500,000g)

The first step in fractionation of the crude Hib bacterial disruptate was a simple centrifugation step (**Section 2.3.4.5.**) to separate soluble cytoplasmic material from cell wall, membrane and other insoluble materials. Initially the ultracentrifugation was carried out at 500,000g, 4°C, 16h and both the supernatant and pellet fractions were assessed for ototoxicity.

4.2.1. Supernatant (I) (cytoplasmic contents (I))

The supernatant (I) fraction of crude Hib bacterial disruptate produced no positive losses in CAPs or CM in one animal (**Fig. 4.2.1**; **Table 4.2.1**). At 90 min, 10kHz CAP loss was 18dB which is just below the significance level.

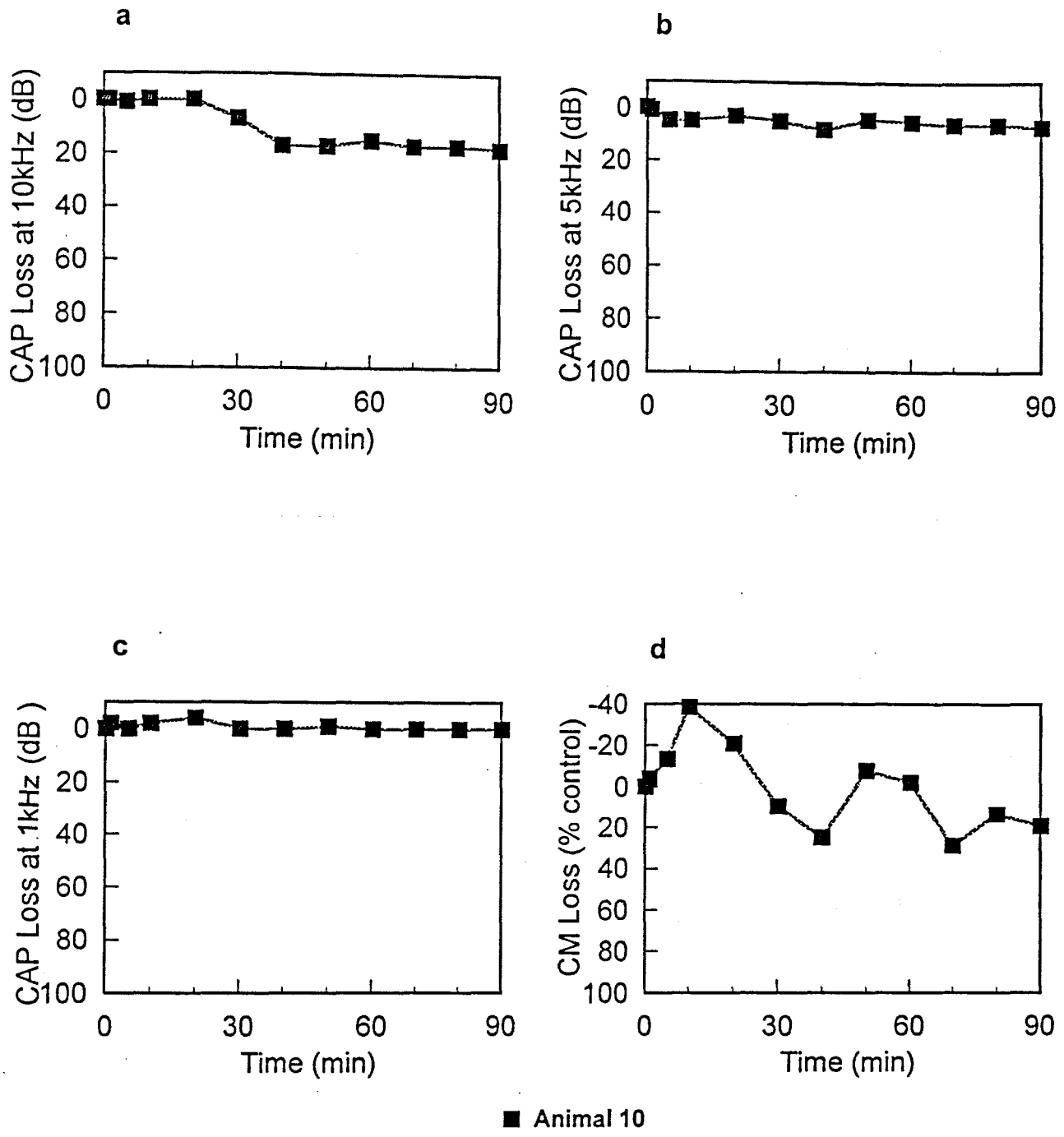


Figure 4.2.1. Losses in the CAP and CM in one animal, treated with cytoplasmic contents of crude Hib bacterial disruptate.

a-c) Losses in the CAP elicited with 10, 5 and 1kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

Table 4.2.1 : Electrophysiological losses evoked by perfusion with supernatant (I) fraction of Hib crude bacterial disruptate in one animal. Positive losses are bold.

Time post perfusion (min)	Animal 10			
	CAP			CM
	10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) Peak CM (mV)			
	8	4	45	0.18
	Losses in CAPs (dB) and CM (% control)			
1	0	1	-2	-4
5	1	5	0	-13
10	0	5	-2	-38
30	7	5	0	10
60	15	5	0	-2
90	18	7	0	19

Conclusions

- There was little or no evidence for ototoxicity in the supernatant fraction (I) of crude Hib bacterial disruptate. The pellet fraction (I) was not tested for ototoxic activity as we wished to reconsider the ultracentrifugation method before continuing.

4.3 Ultracentrifugation II (30min at 500,0000g)

Previous evidence (Amaee 1995) suggested that the Hib ototoxic factor was present in the soluble cytoplasmic contents of crude Hib bacterial disruptate. The initial ultracentrifugation (section 4.2) produced a supernatant fraction which displayed some, but considerably reduced ototoxicity (as judged by CAP losses) in comparison to the crude extract, indicating perhaps that some of the ototoxicity had been sedimented into the pellet fraction. Since we wished to distinguish between ototoxic activity that may be produced by LPS and that of any other ototoxic factors we decided to modify the ultracentrifugation method in an attempt to eliminate/reduce any sedimentation of the ototoxic factor into the pellet (which will contain LPS). In all future fractionations ultracentrifugation was carried out at 500,000g for 30 min as apposed to 16 h to retain relatively more of the 'pelleted' material in the supernatant. Both the supernatant and pellet fractions of the crude Hib bacterial disruptate were assessed for ototoxicity.

4.3.1 Supernatant (II) (cytoplasmic contents (II))

The supernatant (II) fraction of crude Hib bacterial disruptate was tested for ototoxicity in four animals (**Fig. 4.3.1**; **Table 4.3.1**) and produced large CAP losses at all frequencies. At 90 min, 10kHz CAP losses were 61, 75, 68 and 46 dB in animals 11, 12, 13 and 14 respectively. Positive CM losses were displayed in animals 12 and 14, but not animals 11 and 13, although animal 11 displayed a loss in the CM of 37% which is just below the significance level.

Table 4.3.1 : Electrophysiological losses evoked by perfusion with Hib cytoplasmic contents in four animals.
Positive losses are bold.

Time post perfusion (min)	Animal 11				Animal 12			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	11	-1	41	0.33	5	-4	45	0.11
	Loss in CAPs (dB) or CM (% control)							
1	9	11	7	9	0	9	0	-6
5	**34**	**49**	**44**	7	**55**	**46**	**23**	3
10	**55**	**74**	**46**	14	**70**	**62**	**35**	22
30	**61**	**91**	**64**	18	**70**	**72**	**33**	0
60	**61**	**91**	**64**	31	**75**	**74**	**37**	19
90	**61**	**91**	**64**	37	**75**	**76**	**46**	**44**

Time post perfusion (min)	Animal 13				Animal 14			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	12	10	45	0.28	35	31	57	0.14
	Loss in CAPs (dB) or CM (% control)							
1	1	-2	-1	3	**23**	**22**	**21**	**44**
5	**21**	15	10	-12	**36**	**26**	**26**	**54**
10	**54**	**52**	**29**	-12	**36**	**26**	**25**	**61**
30	**63**	**65**	**30**	0	**41**	**32**	**32**	**71**
60	**68**	**68**	**38**	10	**47**	**37**	**31**	**61**
90	**68**	**68**	**38**	7	**46**	**39**	**31**	**71**

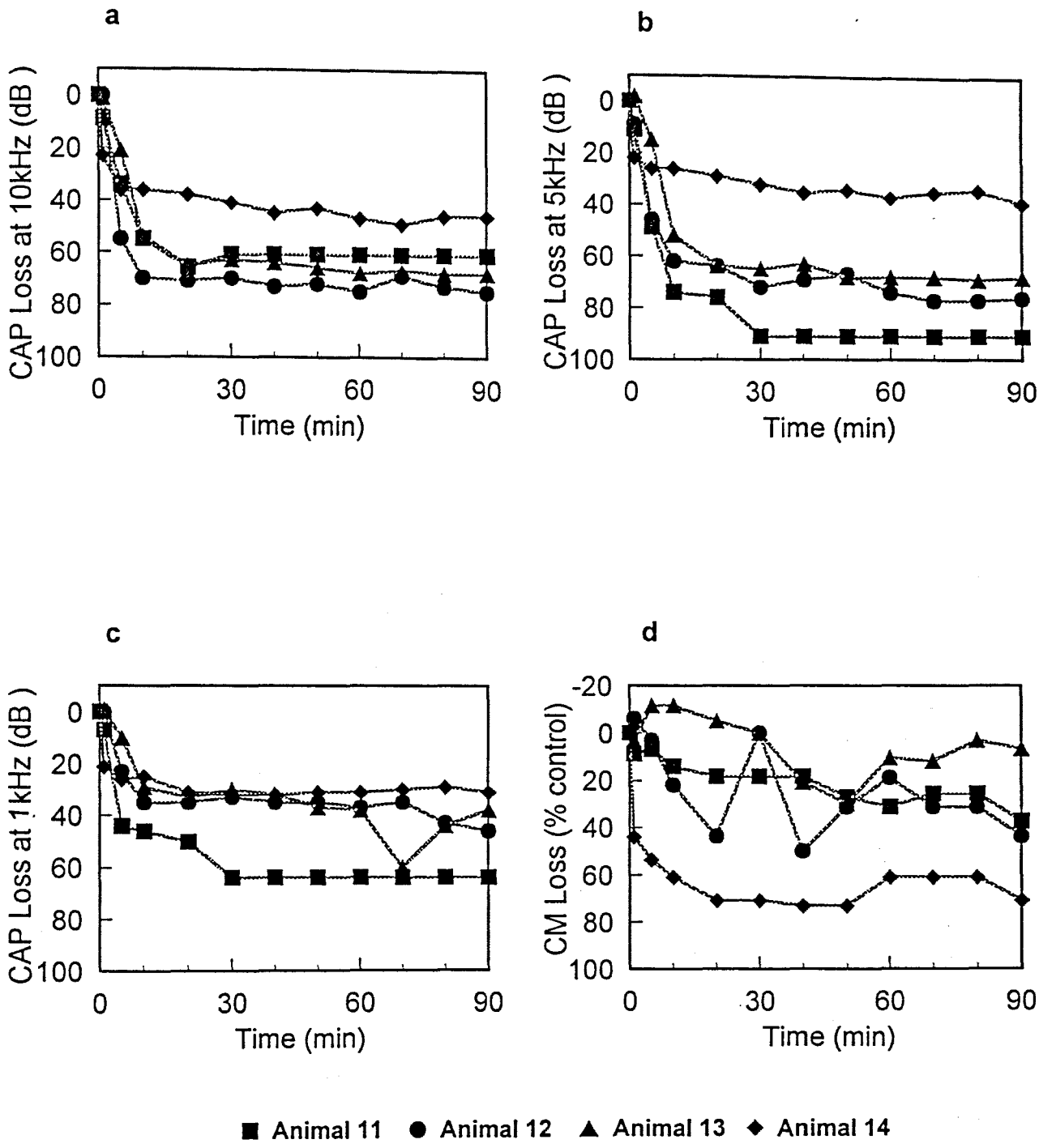


Figure 4.3.1. Losses in the CAP and CM in four animals, each treated with Hib cytoplasmic contents (II).

a-c) Losses in the CAP elicited with 10, 5 and 1 kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

4.3.2 Pellet Fraction (II) (cell wall and membrane material (II))

The pellet fraction of the crude Hib bacterial disruptate was tested for ototoxicity in animals 15, 16 and 17 (**Fig. 4.3.2**; **Table 4.3.2**). The data from animal 17 are included in Fig. 4.3.2 and Table 4.3.2, but are excluded from statistical and summary data as the animal displayed initial auditory thresholds outside the defined range. Animals 16 and 17 displayed no positive losses in CAPs (losses did not exceed 8dB). In contrast animal 15 displayed positive CAP losses at all frequencies and at all time points following perfusion. In all animals there were no positive losses in the CM.

Table 4.3.2: Electrophysiological losses evoked by perfusion with unwashed Hib cell wall and membrane material in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 15				Animal 16				Animal 17			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	38	12	45	0.32	21	12	46	0.21	61	40	67	0.27
	Loss in CAPs (dB) or CM (% control)											
1	**24**	**37**	**32**	-1	-2	3	0	-4	1	2	0	0
5	**28**	**44**	**32**	-4	1	-2	2	-2	0	2	-2	-2
10	**31**	**47**	**31**	-4	4	0	-1	-4	2	-2	-4	21
30	**30**	**53**	**34**	2	4	3	0	-4	2	0	-2	-2
60	**31**	**50**	**35**	4	4	3	2	0	5	3	-2	33
90	**34**	**55**	**36**	9	7	8	2	-2	6	5	-1	8

4.3.3. Washed Pellet Fraction (Cell Wall And Membrane Material)

There was some evidence for ototoxicity in the pellet fraction of the crude Hib bacterial disruptate. However the results were not consistent between animals. It is possible that the ototoxicity produced by the pellet fraction was due to contamination of the sample with some components of the supernatant which is potently ototoxic (as judged by CAP losses >40dB see Section 4.3.1). Therefore the pellet sample produced by ultracentrifugation at 500,000g was washed twice with APL and reassessed for ototoxicity in animals 18, 19 and 20 (**Fig. 4.3.3**; **Table 4.3.3**). Perfusion of the washed pellet fraction produced no positive CAP losses in animal 18, but some positive CAP losses in animals 19 and 20. At 90 min post perfusion animal 19 exhibited a

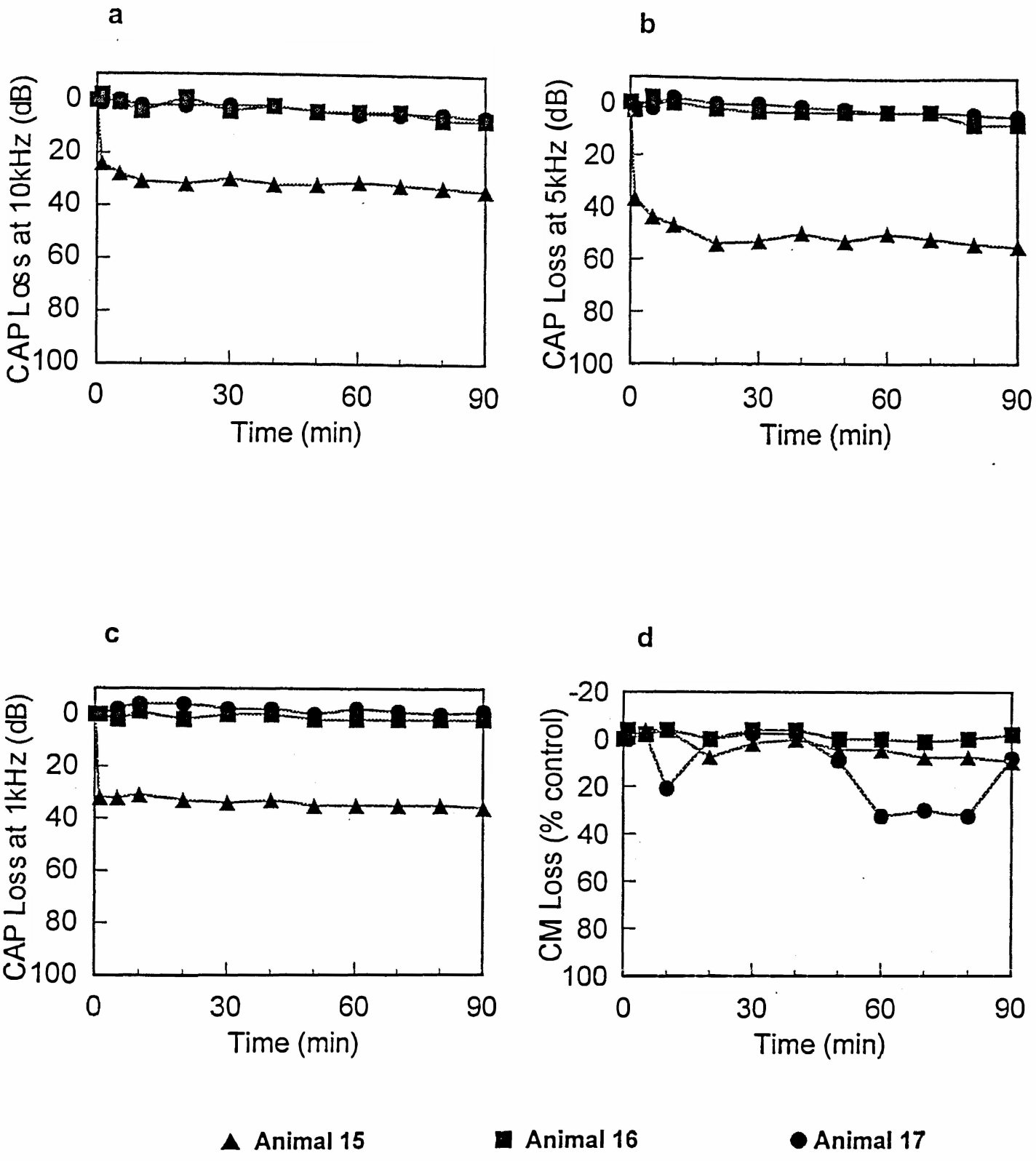


Figure 4.3.2. Losses in the CAP and CM in four animals, each treated with Hib cell wall and memrane material.

a-c) Losses in the CAP elicited with 10, 5 and 1 kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

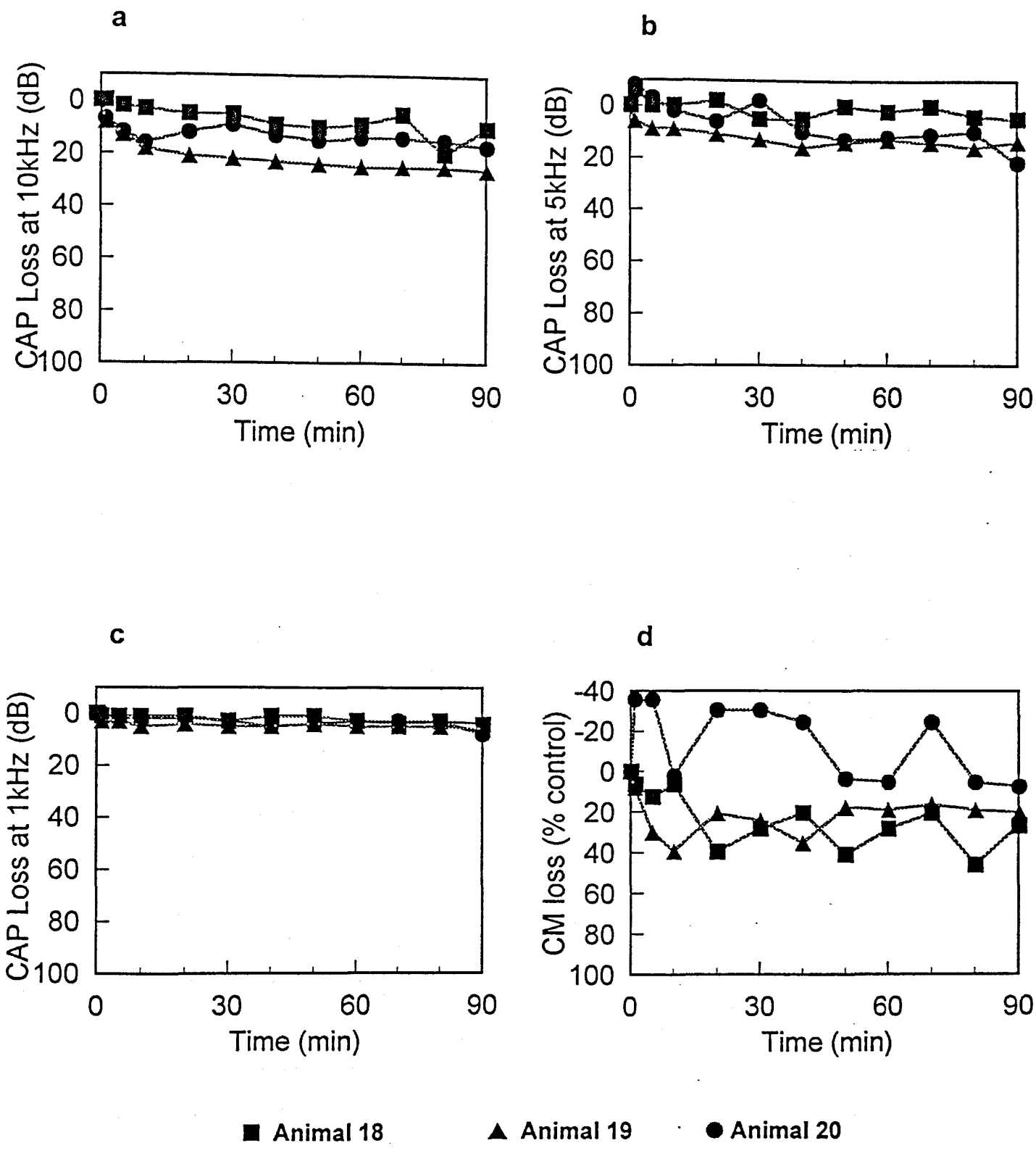


Figure 4.3.3. **Losses in the CAP and CM in three animals, each treated with washed Hib cell wall and membrane material.**

a-c) Losses in the CAP elicited with 10, 5 and 1 kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

loss of 26dB in the 10kHz CAP, and animal 20 exhibited a loss of 22dB in the 5kHz CAP. In all animals there were no positive losses in the CM.

Table 4.3.3: Electrophysiological losses evoked by perfusion with washed Hib cell wall and membrane material in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 18				Animal 19				Animal 20			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	20	10	44	0.23	15	-4	38	0.35	8	-7	40	0.28
	Loss in CAPs (dB) or CM (% control)											
1	0	-6	1	6	8	6	3	8	7	-8	0	-36
5	2	0	1	12	13	9	3	30	12	-3	2	-36
10	3	0	1	6	18	9	5	39	16	2	2	2
30	5	5	3	28	**22**	13	5	24	9	-2	3	-30
60	9	2	3	28	**25**	13	5	19	14	12	3	5
90	10	5	4	27	**26**	14	6	20	17	**22**	8	7

4.3.4. Ultracentrifugation II (30 min) : Summary and Conclusions

Average 90 min losses in CAPs and CMs displayed by animals perfused with the fractions produced by ultracentrifugation at 500,000g, 30 min, 4°C are summarised in **Table 4.3.4.**

Table 4.3.4 : Average 90 min losses evoked by perfusion with Crude Hib disruptate, and fraction products of ultracentrifugation at 500,000g, 30 min, 4°C. Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 90 min post perfusion			
	CAP at 10 kHz	CAP at 5 kHz	CAP at 1kHz	CM
Crude Hib Disruptate (n=3)	**49 ± 27**	**35 ± 26**	**22 ± 13**	32 ± 20
Supernatant (n=4)	**62 ± 12**	**68 ± 22**	**45 ± 14**	**40 ± 26**
Unwashed Pellet (n=3)	**20 ± 19**	**31 ± 33**	19 ± 24	4 ± 8
Washed Pellet (n=3)	18 ± 8	14 ± 8	6 ± 2	18 ± 10

Conclusions

- Cytoplasmic contents of the crude Hib bacterial disruptate were potently ototoxic (as judged by CAP loss >40dB) in the cochlea. Both unwashed and washed pellet fractions (derived from crude Hib bacterial disruptate) produced some low levels of ototoxicity (as judged by CAP loss >20dB) in the cochlea, but the results were not consistent between animals.

- Statistical analysis using ANOVA demonstrated a significant difference in the 10kHz CAP losses between the supernatant fraction (derived from crude Hib bacterial disruptate) and either the washed or unwashed pellet fractions (derived from crude Hib bacterial disruptate) over time. The effect of treatment alone was significant ($p=0.02$ and $p=0.03$ for the washed and unwashed pellet fractions respectively) and the effect of treatment over time was highly significant ($p=<0.0001$ in either case). There was no significant difference in the 10kHz CAP losses between the washed and unwashed pellet fractions.

- The results indicated that the majority of the ototoxic activity was associated with the cytoplasmic contents of the crude Hib bacterial disruptate (when ultracentrifuged for 30 min at 500,000g, 4°C) and hence this extract was used for further fractionation.

- The cell wall and membranous material produced some low-level ototoxicity. Since purified *E. coli* LPS produces some ototoxicity in the guinea pig cochlea (Comis *et al.* 1991) it is possible that low level ototoxicity produced by the pellet fraction may have been due to LOS which would have been present in considerable amounts within this fraction. This was not further examined.

4.4. Anion Exchange Chromatography (I) (Single Step Elution)

This section was carried out in collaboration with Dr Lynn Dover, whose permission I have to present the combined data as part of my thesis.

The next step in fractionation was to use anion exchange chromatography to separate the cytoplasmic contents of Hib according to their charge. Initially a small column was set up (bed volume approximately 1ml) as described in Methods (**Section 2.3.4.6.**). 15mg aliquots (approximately 1ml) total protein were added to the column. The unadsorbed fraction was collected and the bound sample eluted using 10ml 1M NaCl. All samples were reconcentrated, dialysed in APL, and diluted to a concentration equal to that in the original supernatant material. Both the unadsorbed and adsorbed fractions were assessed for ototoxicity.

Elution Profile

An elution profile was carried out to determine at what point the majority of protein was eluted from the column during this anion exchange chromatography method (**Table 4.4.**).

Table 4.4. Elution Profile : Volumes and Protein Content of fractions of crude Hib cytoplasmic contents produced by anion exchange chromatography.

Eluent	Fraction	Fraction Volume (µl)	Cumulative Volume (µl)	[Protein] (mg/ml)	Protein (µg)	Cumulative Protein (µg)
50 mM Tris.HCl pH 7.5	1	990	990	0.25	247.5	247.5
	2	1200	2190	1.46	1752	1999.5
	3	860	3050	0.45	387	2386.5
	4	590	3640	0.2	118	2504.5
	5	950	4590	0.31	294.5	2799
	6	930	5520	0.21	111.6	2910.6
	7	815	6335	0.07	57.05	2967.65
	8	900	7235	0.18	162	3129.65
	9	1020	8255	0.07	71.4	3201.05
50 mM Tris.HCl pH 7.5 + 1M NaCl	10	1160	9415	1.55	1798	4999.05
	11	846	10261	8.5	7191	12190.05
	12	620	10881	0.54	334.8	12524.85
	13	1300	12181	0.02	26	12550.85
	14	1200	13381	0.02	24	12574.85
	15	890	14271	0.02	62.3	12637.15

The Tris HCl eluted fraction (unadsorbed sample) contained 3mg of protein and the 1M NaCl eluted fraction (adsorbed sample) contained 9mg of protein (12mg in total). No further protein was eluted by 4M LiCl. Therefore it was concluded that all of the protein was eluted using 50ml 1M NaCl, the majority of it having adsorbed to the column and hence this method was used to attempt to isolate the ototoxic element in the Hib cytoplasmic contents.

4.4.1. Unadsorbed Fraction

The unadsorbed fraction of the Hib cytoplasmic contents was tested for ototoxicity in animals 21 and 22 (**Fig. 4.4**; **Table 4.4.1**), and produced no positive losses in CAPs or CM. However, in animal 21 positive increases in the 5kHz CAP occurred.

Table 4.4.1 : Electrophysiological losses evoked by perfusion with unadsorbed fraction of crude Hib cytoplasmic contents in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 21				Animal 22			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	17	18	48	0.09	28	40	61	0.21
	Loss in CAPs (dB) or CM (% control)							
1	8	7	3	-10	2	-24	-9	8
5	9	8	3	8	2	-20	-9	13
10	9	5	4	4	2	-20	-8	1
30	9	6	2	8	2	-21	-10	34
60	10	8	5	-8	4	-20	-10	5
90	12	11	6	4	4	-19	-9	6

4.4.2. 1M NaCl Fraction

The adsorbed fraction of the Hib cytoplasmic contents eluted using 1M NaCl was tested for ototoxicity in animals 23 and 24 (**Fig. 4.4**; **Table 4.4.2**), and produced positive losses in the 10

and 5kHz CAPs in both animals. Animal 23 displayed a positive CAP loss at all frequencies within 5 min of perfusion. A positive loss in the CM was seen in animal 23 at 90 min, but not in animal 24.

Table 4.4.2 : Electrophysiological losses evoked by perfusion with 1M NaCl fraction of crude Hib cytoplasmic contents in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 23				Animal 24			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	49	34	51	0.14	30	13	51	0.29
	Loss in CAPs (dB) or CM (% control)							
1	12	**26**	17	27	-1	-1	-1	-5
5	**21**	**34**	**22**	2	13	0	1	8
10	**22**	**35**	**24**	20	13	-1	0	4
30	**25**	**34**	**25**	25	15	7	1	3
60	**26**	**34**	**26**	32	**22**	15	11	10
90	**26**	**33**	**26**	**55**	**28**	**22**	13	16

4.4.3. Anion Exchange Chromatography I (Single Step Elution) : Summary and Conclusions

Average 90 min losses in CAPs and CMs displayed by animals perfused with the fractions produced by 'Anion exchange chromatography I' are summarised in **Table 4.4.3.**

Table 4.4.3 : Average 90 min losses evoked by perfusion with fractions from 'Anion exchange chromatography I'. Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 90 min post perfusion			
	CAP at 10 kHz	CAP at 5 kHz	CAP at 1kHz	CM
Unadsorbed Fraction (n=2)	8 ± 6	-4 ± 21	-1 ± 11	5 ± 2
1MNaCl Fraction (n=2)	**27 ± 1**	**27 ± 8**	19 ± 9	35 ± 28

Conclusions

- There was evidence of ototoxicity in the 1M NaCl fraction. The unadsorbed fraction produced some moderate ototoxicity (as judged by CAP loss), however this was not consistent between animals.
- Statistical analysis using ANOVA demonstrated a significant difference between the 10kHz CAP losses produced by the unadsorbed and 1M NaCl fractions over time. The effect of treatment on the CAP at all time points was not significant ($p=0.09$), but the change in the CAP with time was significant ($p=0.001$).
- The results indicated that the majority of the ototoxic activity was in the 1M NaCl fraction of the cytoplasmic contents of Hib, and hence this fraction was further fractionated.

4.5. Anion Exchange Chromatography II (Two-Step Elution)

A column, similar to that described in section 4.4, was loaded in an identical fashion. The column was desorbed in a 2 step fashion using 50mM Tris.HCl pH7.5 (10 ml/step) containing 0.2M and 1M NaCl respectively. Fractions were pooled appropriately and tested for ototoxicity

4.5.1 Unadsorbed Fraction (II)

The unadsorbed fraction (II) of Hib cytoplasmic contents was again tested for ototoxicity in animals 25 and 26 (**Fig. 4.5 i; Table 4.5.1**), and produced no losses in CAPs or CMs. CAP losses did not exceed 11 dB.

Table 4.5.1 : Electrophysiological losses evoked by perfusion with unadsorbed fraction of crude Hib cytoplasmic contents in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 25				Animal 26			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	24	19	48	0.14	6	10	43	0.14
	Loss in CAPs (dB) or CM (% control)							
1	4	1	-1	-36	9	0	4	-10
5	4	3	3	-36	4	5	0	-10
10	5	5	4	-38	9	7	0	-7
30	10	10	3	-20	7	8	2	-17
60	10	11	6	15	7	7	0	-17
90	8	8	5	-15	10	8	0	-12

4.5.2 0.2M NaCl Fraction

The fraction of the Hib cytoplasmic contents eluted with 0.2M NaCl was tested for ototoxicity in animals 27 and 28 (**Fig. 4.5 ii; Table 4.5.2**). Animal 27 displayed positive CAP losses at 10 and 5 kHz, whereas CAP losses displayed by animal 28 did not exceed 14dB. CM losses were not positive in either animal.

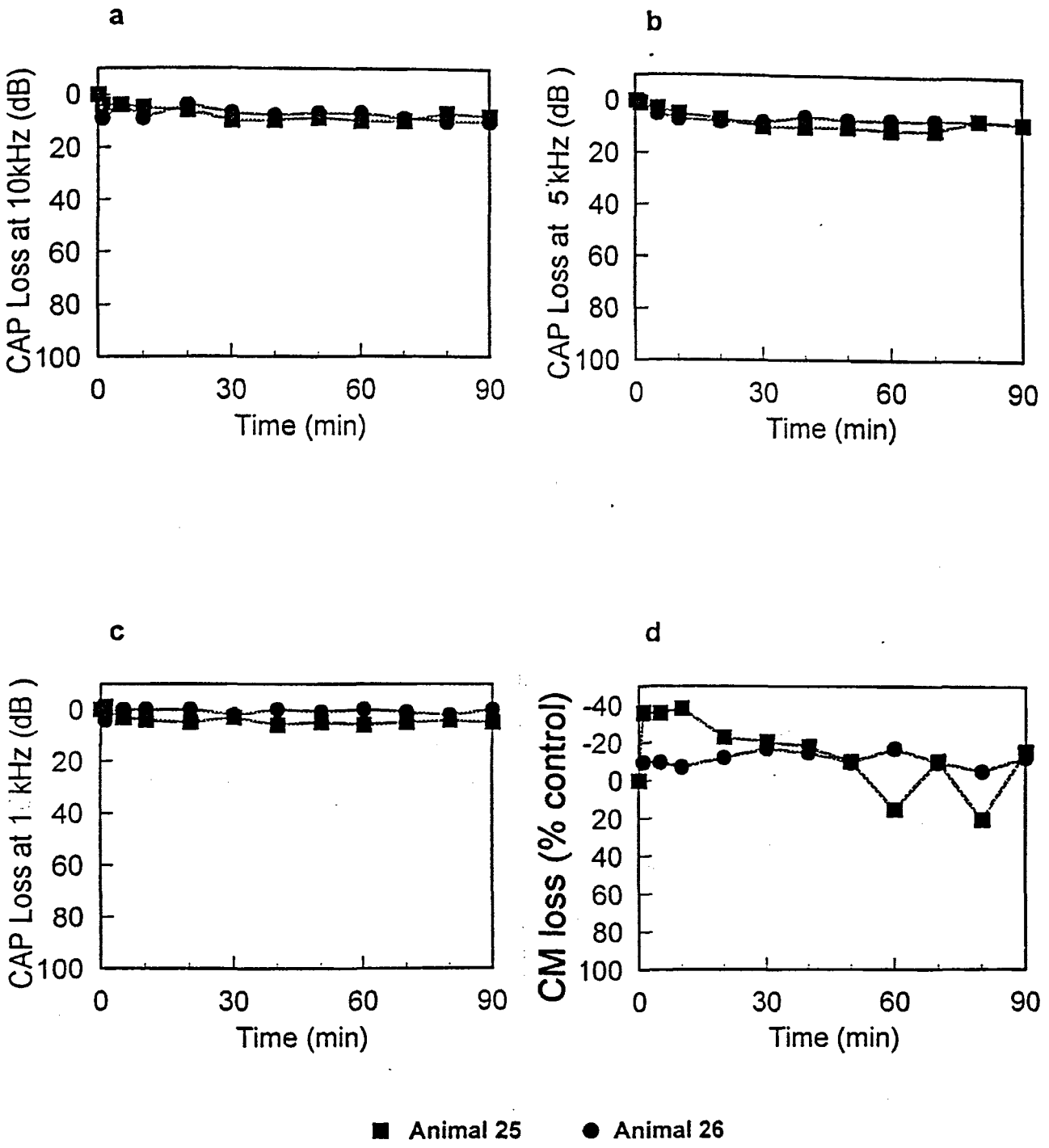


Figure 4.5 i. Losses in the CAP and CM in two animals each treated with unadsorbed fraction (II) of Hib cell wall cytoplasmic contents .

a-c) Losses in the CAP elicited with 10, 5 and 1 kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

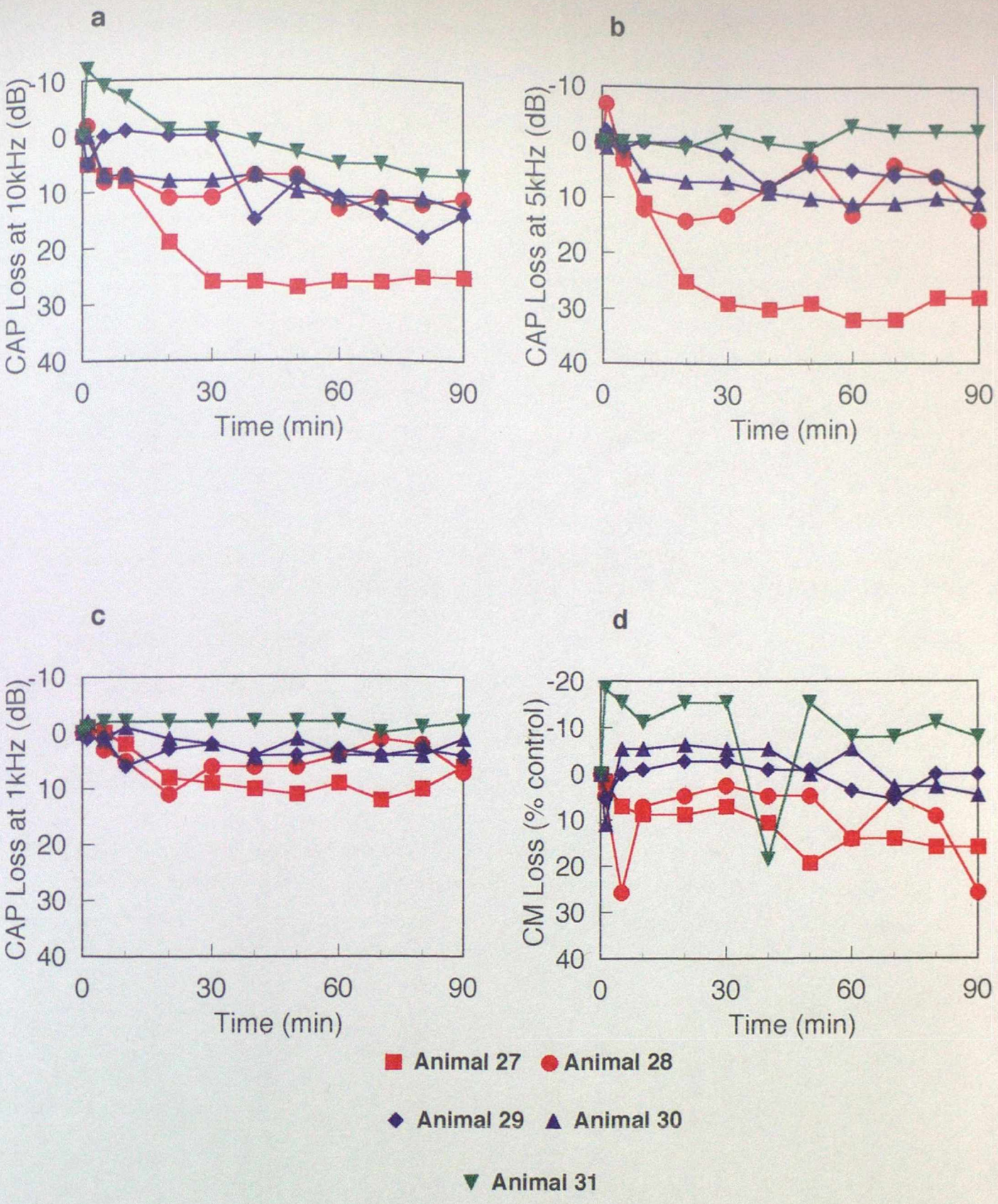


Figure 4.5 ii. **Losses in the CAP and CM in five animals, two treated with 0.2M NaCl fraction (RED), two treated with 1M NaCl fraction (BLUE) and one treated with a mixture of the 0.2M and 1M NaCl fractions (GREEN) of the Hib cytoplasmic contents.**

a-c) Losses in the CAP elicited with 10, 5 and 1kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

Table 4.5.2 : Electrophysiological losses evoked by perfusion with 0.2M NaCl fraction of crude Hib cytoplasmic contents in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 27				Animal 28			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 perfusion	Threshold CAPs (dB) and Peak CM (mV)							
	27	2	43	0.20	28	30	52	0.15
	Loss in CAPs (dB) or CM (% control)							
1	5	-1	-1	2	-2	-7	-1	5
5	7	3	0	7	8	3	3	26
10	8	11	2	9	7	12	5	7
30	**26**	**29**	9	7	11	13	6	2
60	**26**	**32**	9	14	13	13	4	14
90	**25**	**28**	6	16	11	14	7	26

4.5.3 1M NaCl Fraction

The 1M NaCl fraction of Hib cytoplasmic contents eluted with 1M NaCl was tested for ototoxicity in animals 29 and 30 (**Fig. 4.5 ii**; **Table 4.5.3**), and produced no positive losses in CAPs or CMs. CAP losses did not exceed 14dB.

Table 4.5.3: Electrophysiological losses evoked by perfusion with 1M NaCl fraction of crude Hib cytoplasmic contents in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 29				Animal 30			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	2	14	40	0.22	21	9	46	0.22
	Loss in CAPs (dB) or CM (% control)							
1	5	-2	1	5	0	1	-2	11
5	0	1	1	0	7	0	1	-5
10	-1	0	6	-1	7	6	-1	-5
30	0	2	2	-3	8	7	2	-5
60	11	5	3	4	11	11	4	-5
90	14	9	4	0	13	11	1	5

4.5.4 Mixture of 0.2M And 1M NaCl Eluted Fractions

The 0.2M and 1M NaCl fractions were mixed and assessed for ototoxicity to test the hypothesis that the ototoxic factor required two or more components which may have been separated during this fractionation step. Since the 0.2M NaCl fraction was itself ototoxic, the expectation was for an increased ototoxicity in the mixture if two components are necessary. 500µl of each 0.2M and 1M NaCl fractions were mixed overnight at 4 °C, and then tested for ototoxicity in animal 31 (**Fig. 4.5 ii; Table 4.5.4**). No positive losses in CAPs or CM were seen. CAP losses did not exceed 7dB.

Table 4.5.4: Electrophysiological losses evoked by perfusion with a mixture of the 0.2M and 1M NaCL fractions of crude Hib cytoplasmic contents in one animal.

Positive losses are bold.

Time post perfusion (min)	Animal 31			
	CAP			CM
	10 kHz	5 kHz	1kHz	
0 (control)	Threshold CAPs (dB) Peak CM (mV)			
	22	14	41	0.19
	Losses in CAPs (dB) and CM (% control)			
1	-12	0	-1	-19
5	-9	0	-2	-15
10	-7	0	-2	-11
30	-1	-2	-2	-15
60	5	-3	-2	-8
90	7	-2	-2	-8

4.5.5. Anion Exchange Chromatography II (Two-Step Elution) : Summary and Conclusions

Average 90 min losses in CAPs and CMs displayed by animals perfused with the fractions produced by 'Anion exchange chromatography II' are summarised in **Table 4.5.5.**

Table 4.5.5 : Average 90 min losses evoked by perfusion with produced by 'Anion exchange chromatography II'. Positive losses are bold.

Treatment	Average losses (± SD) in the CAPs (dB) or CM (%) at 90 min post perfusion			
	CAP at 10 kHz	CAP at 5 kHz	CAP at 1kHz	CM
0.2M NaCl Fraction(n=2)	18 ± 10	**21 ± 10**	6 ± 1	21 ± 7
1M NaCl Fraction(n=2)	13 ± 1	10 ± 1	2 ± 2	2 ± 3
0.2 & 1M NaCl Fractions (n=1)	7	-2	-2	-8

Conclusions

- There was evidence for ototoxicity in the 0.2M NaCl fraction, however the results were not consistent between animals. The unadsorbed fraction and 1M NaCl fraction produced no ototoxicity in the guinea pig cochlea. Statistical analysis using ANOVA demonstrated no significant difference between the 10kHz CAP losses produced by the unadsorbed, 1M NaCl, and 0.2M NaCl.
- The mixture of 0.2M and 1M NaCl fractions displayed no increase in ototoxicity. Since only one animal was used any conclusions must be highly tentative. According to the results it would appear there was no ototoxin-enhancing component present in the 1M NaCl fraction, or the toxin activity in the 0.2M NaCl fraction had been diluted or neutralised.
- The level of ototoxicity (as judged by CAP loss) in fractions produced by anion exchange chromatography was considerably lower than seen in crude fractions. Therefore it appeared that some activity was being lost during this fractionation step. This loss of activity was not due to separation of two or more individual factors required for the ototoxic activity.

4.6. Anion Exchange Chromatography III (Two-Step Elution)

In order to limit any breakdown of the ototoxic factor potentially due to co-purification with proteases, samples were bulked with 10mg bovine serum albumin (BSA) per eluate. In this way if there were any protease/s present within the fractions BSA would contribute the majority of the available substrate. The column was set up and loaded as in 4.5 (anion exchange chromatography II), and all adsorbed fractions were assessed for ototoxicity.

4.6.1 0.2M NaCl Fraction Bulked With BSA.

The adsorbed fraction of the Hib cytoplasmic contents eluted with 0.2M NaCl which was bulked with BSA was tested for ototoxicity in animals 32 and 33 (**Fig. 4.6**; **Table 4.6.1**), and produced positive CAP losses at 10 and 5kHz in both animals. Data from animal 32 are included in Fig. 4.6 and Table 4.6.1, but are excluded from statistical and summary data as the animal died at 50 min. Final 10kHz CAP losses were 31 and 35dB in animals 32 and 33 respectively. Neither animal displayed positive CM losses.

Table 4.6.1 : Electrophysiological losses evoked by perfusion with 0.2M fraction of crude Hib cytoplasmic contents bulked with BSA in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 32				Animal 33			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	25	0	49	0.08	15	0	44	0.15
	Loss in CAPs (dB) or CM (% control)							
1	6	6	2	0	0	-2	0	-39
5	14	16	6	32	5	2	1	-36
10	14	16	9	0	11	8	3	-36
30	**23**	**24**	13	14	**50**	**49**	**26**	-33
40	**31**	**33**	14	4	**42**	**48**	**33**	-36
60	-	-	-	-	**51**	**59**	**29**	-46
90	-	-	-	-	**35**	**40**	15	-46

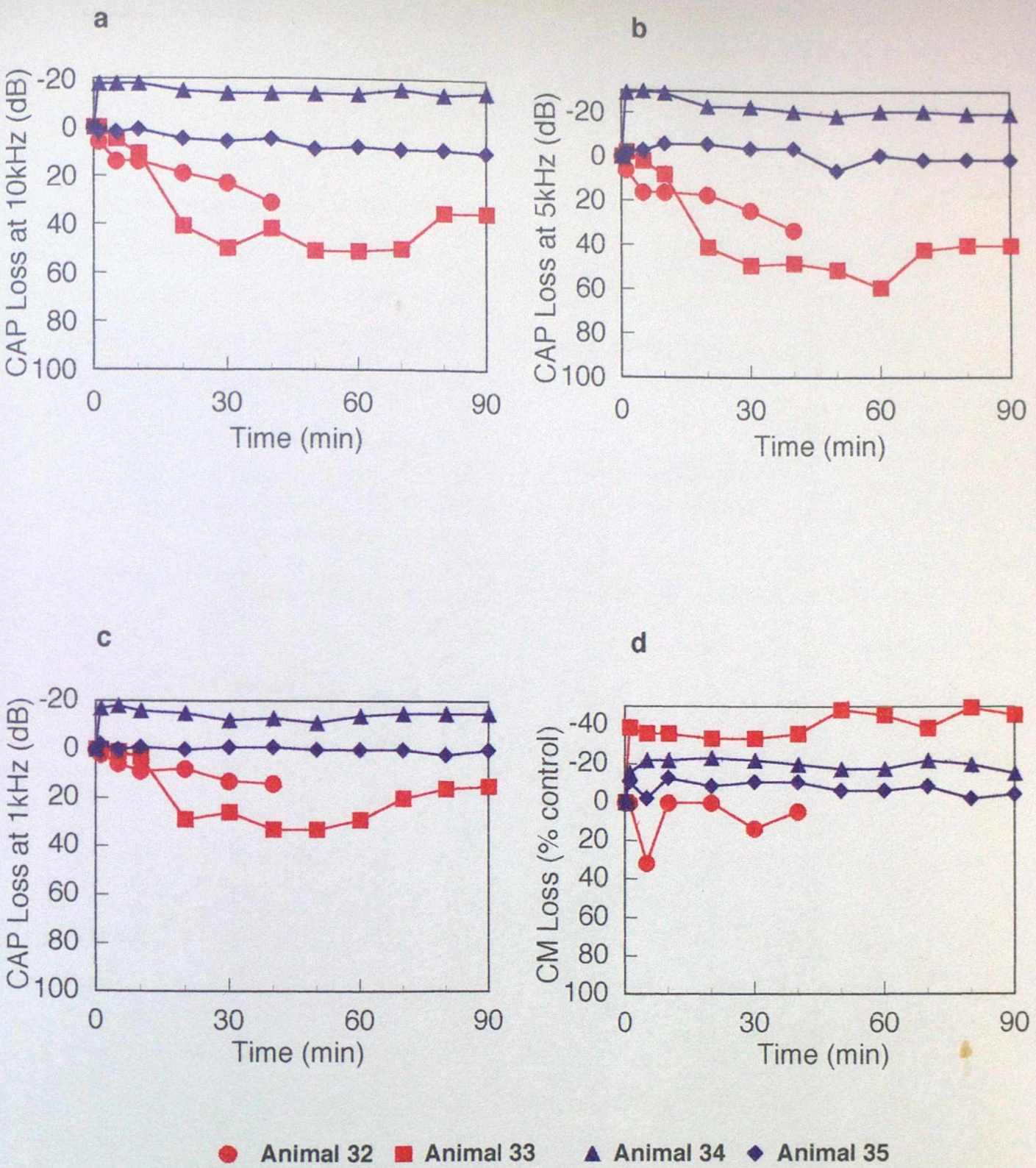


Figure 4.6. Losses in the CAP and CM in four animals, two treated with 0.2M NaCl fraction (RED) and two treated with 1M NaCl fraction (BLUE) of Hib cytoplasmic contents all bulked with BSA.

a-c) Losses in the CAP elicited with 10, 5 and 1kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

4.6.2 1M NaCl Fraction Bulked with BSA

The adsorbed fraction of the Hib cytoplasmic contents eluted with 1M NaCl was tested for ototoxicity in animals 34 and 35 (**Fig. 4.6**; **Table 4.6.2**), and produced no positive losses in CAPs or CMs. However, positive CAP increases and some CM increases were displayed in animal 34, which are likely to have been due to poor initial electrical coupling (**discussed in section 3.1**). Nonetheless, recalculation of CAP and CM losses using 1 min recordings as control values, reveals no positive losses in CAPs or CM.

Table 4.6.2 : Electrophysiological losses evoked by perfusion with 1M NaCl fraction of crude Hib cytoplasmic contents bulked with BSA in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 34				Animal 35			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	1 kHz		10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	42	33	61	0.18	23	12	45	0.16
	Loss in CAPs (dB) or CM (% control)							
1	-18	-29	-17	-15	2	-2	-2	-11
5	-18	-30	-18	-22	2	-3	0	-2
10	-18	-29	-16	-22	1	-6	-1	-13
30	-14	-23	-12	-22	6	-4	-1	-11
60	-14	-21	-14	-18	8	-1	0	-6
90	-15	-20	-15	-15	10	1	0	-4

4.6.3. Anion Exchange Chromatography III (Two-Step Elution) : Summary and Conclusions

Average 90 min losses in CAPs and CMs displayed by animals perfused with the fractions produced by 'Anion exchange chromatography III' (bulked with BSA) are summarised in **Table 4.6.3.**

Table 4.6.3 : Average 90 min losses evoked by perfusion with produced by 'Anion exchange chromatography III'. Positive losses are bold.

Treatment	Average losses (± SD) in the CAPs (dB) or CM (%) at 90 min post perfusion			
	CAP at 10 kHz	CAP at 5 kHz	CAP at 1kHz	CM
0.2M NaCl Fraction (n=1)	**35**	**40**	15	-46
1M NaCl Fraction (n=2)	-2 ± 18	-9 ± 15	-7 ± 11	-10 ± 8

Conclusions

- There was evidence of ototoxicity in the 0.2M NaCl eluted fraction and not in the 1M NaCl eluted fraction. Statistical analysis was not carried out as data from animal 32 had been excluded.
- The lack of ototoxicity produced by the 1M NaCl fraction meant that any ototoxicity produced by samples bulked with BSA could safely be attributed to ototoxicity in the Hib fraction rather than to BSA.
- The results indicated that the majority of the ototoxic activity was in the 0.2M eluted fraction, and bulking the sample with BSA enhanced maintenance of activity. Hence this fraction was further fractionated and samples were routinely bulked with BSA.

4.7. Anion Exchange Chromatography IV (Three-Step Elution)

A column, similar to that described in section 4.6, was loaded in an identical fashion. The first active desorption step was carried out using Tris.HCl buffer containing 0.1M NaCl. This was followed as before with a 0.2M NaCl elution step. All unadsorbed and adsorbed fractions were assessed for ototoxicity.

4.7.1 Unadsorbed Fraction

The unadsorbed fraction of the Hib cytoplasmic contents fraction produced no positive losses in CAPs or CM in animal 36 (**Fig. 4.7 i; Table 4.7.1**).

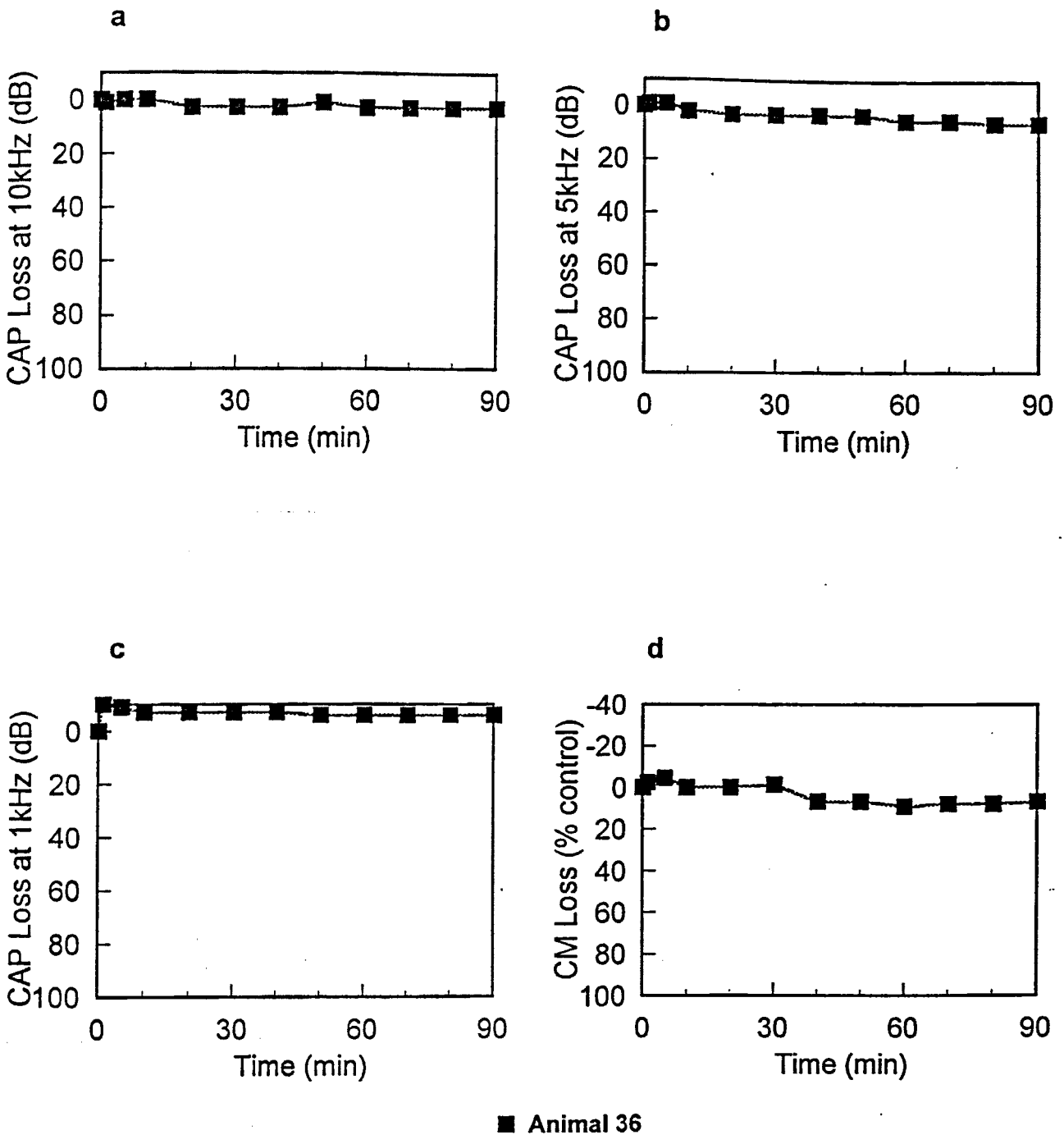


Figure 4.7 i. Losses in the CAP and CM in one animal, treated with unadsorbed fraction of the Hib cytoplasmic contents .

a-c) Losses in the CAP elicited with 10, 5 and 1kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

Table 4.7.1 : Electrophysiological losses evoked by perfusion with unadsorbed fraction of crude Hib cytoplasmic contents bulked with BSA in one animal. Positive losses are bold.

Time post perfusion (min)	Animal 36			
	CAP			CM
	10 kHz	5 kHz	1 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)			
	34	24	65	0.15
	Losses in CAPs (dB) and CM (% control)			
1	1	-1	-10	-2
5	0	-1	-9	-5
10	0	2	-7	0
30	3	3	-7	-1
60	3	5	-6	9
90	3	6	-6	7

4.7.2 0.1M NaCl Fraction

The 0.1M NaCl fraction produced no positive losses in CAPs or CMs in either of animals 37 and 38 within 80 min. However, at 90 min animal 37 displayed positive losses in CAPs at all frequencies. Neither animal displayed positive CM losses (**Fig. 4.7 ii; Table 4.7.2**).

Table 4.7.2 : Electrophysiological losses evoked by perfusion with the 0.1M fraction of crude Hib cytoplasmic contents bulked with BSA in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 37				Animal 38			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	16	9	34	0.19	17	16	32	0.21
	Loss in CAPs (dB) or CM (% control)							
1	3	-3	1	5	-1	-4	-1	-2
5	3	-1	2	11	0	-5	-5	2
10	7	3	2	7	-1	-5	-5	2
30	7	3	2	17	-2	-2	-3	4
60	7	4	4	7	2	-1	-2	10
90	**44**	**49**	**34**	-8	2	1	-1	10

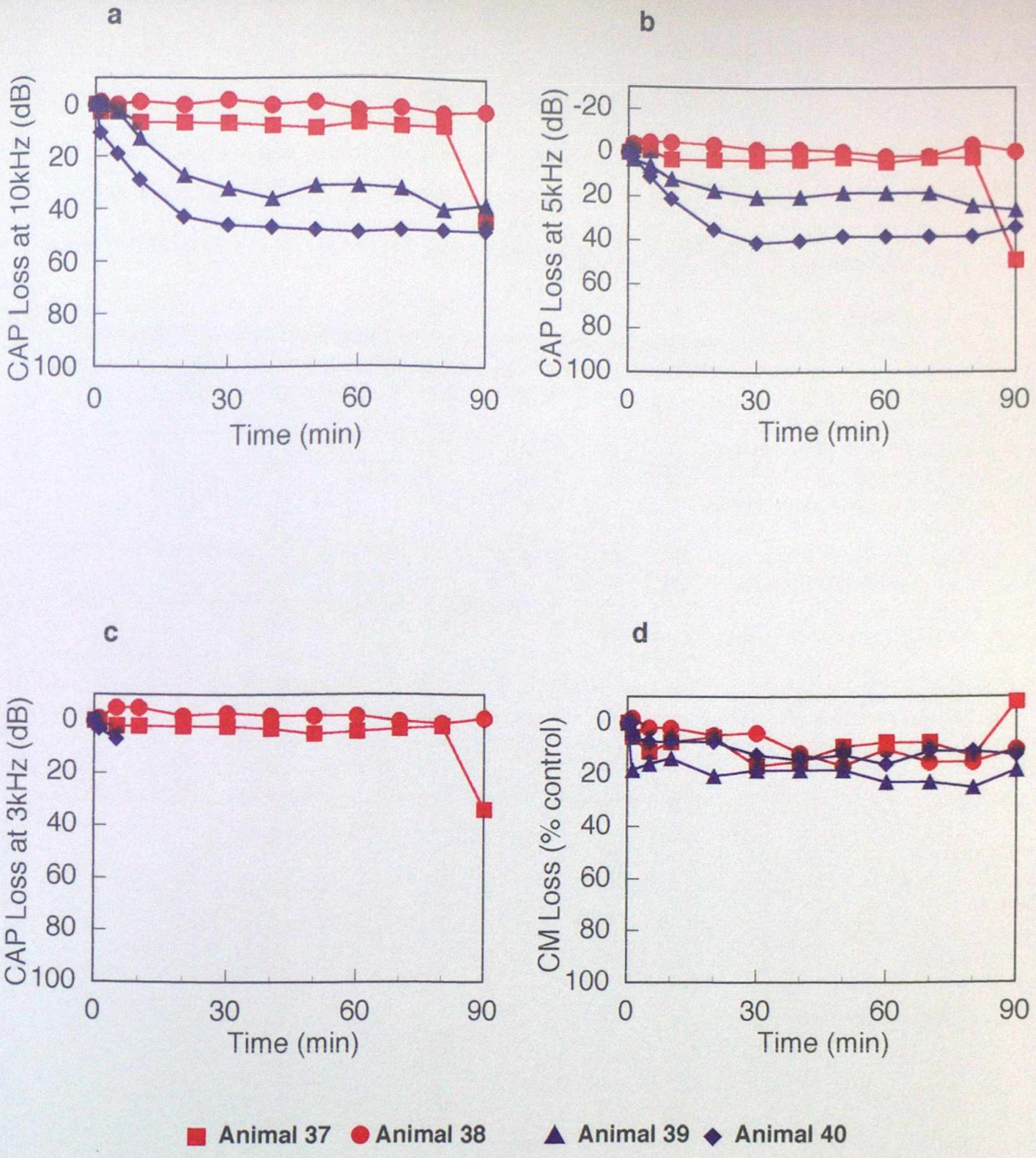


Figure 4.7 ii. Losses in the CAP and CM in four animals, two treated with 0.1M NaCl fraction (RED) and two treated with 0.2M NaCl fraction (BLUE) of Hib cytoplasmic contents all bulked with BSA .

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

4.7.3 0.2M NaCl Fraction

The 0.2M NaCl fraction produced positive losses in the 10 and 5kHz CAPs in both of two animals. At 90 min, 10kHz CAP losses were 38 and 48dB in animals 39 and 40 respectively. A positive loss in the CM was not seen in either animal (**Fig. 4.7 ii; Table 4.7.3**).

Table 4.7.3 : Electrophysiological losses evoked by perfusion with the 0.2M fraction of crude Hib cytoplasmic contents bulked with BSA in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 39				Animal 40			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	29	28	-	0.15	19	17	43	0.20
	Loss in CAPs (dB) or CM (% control)							
1	0	3	-	18	11	2	3	6
5	3	6	-	16	19	11	7	7
10	13	12	-	14	**29**	**21**	-	7
30	**32**	**20**	-	18	**46**	**41**	-	12
60	**31**	18	-	23	**49**	**38**	-	16
90	**38**	**26**	-	18	**48**	**34**	-	12

4.7.4. Anion Exchange Chromatography IV (Three-Step Elution) : Summary and Conclusions

Average 90 min losses in CAPs and CMs displayed by animals perfused with the fractions produced by 'Anion exchange chromatography III' are summarised in **Table 4.7.4.**

Table 4.7.4 : Average 90 min losses evoked by perfusion with produced by 'Anion exchange chromatography IV'. Positive losses are bold.

Treatment	Average losses (± SD) in the CAPs (dB) or CM (%) at 90 min post perfusion (brackets represents losses at 80 min)			
	CAP at 10 kHz	CAP at 5 kHz	CAP at 3kHz	CM
Unadsorbed Fraction (n=1)	3	6	-6	7
0.1M NaCl Fraction (n=2)	**23 ± 30** (5 ± 4)	**24 ± 35** (-1 ± 4)	16 ± 25 (1 ± 1)	1 ± 13 (14 ± 1)
0.2M NaCl Fraction (n=2)	**43 ± 7**	**30 ± 6**	-	15 ± 4

Conclusions

- The unadsorbed fraction of the crude Hib bacterial disruptate produced no ototoxicity in the guinea pig cochlea. There was some evidence for ototoxicity in the 0.1M NaCl fraction. However the results were inconsistent and the temporal pattern of the ototoxicity was not characteristic of that already seen in this work. There was evidence for ototoxicity in the 0.2M eluted fraction.
- Statistical analysis using ANOVA demonstrated a significant difference between the 10kHz CAP losses produced by the 0.1M NaCl and 0.2M NaCl fractions with time. The effect of treatment on the CAP at all time points was not significant ($p=0.08$) but the change in the CAP with time was significant ($p=0.0003$).
- These results indicated that the majority of the ototoxic activity was in the 0.2M eluted fraction.

4.8. Isolation Of The Ototoxicity In Crude Hib Bacterial Disruptate : Summary and Conclusions

Fractionation steps and CAP losses described in this section are summarised in **Fig. 4.8.**

Conclusions

- The majority of the ototoxic activity of the Hib extracts was found in the supernatant fraction following ultracentrifugation at 500,000g, 4^0C for 30 min. This suggests that the ototoxic factor is soluble.

- By combined ultracentrifugation and anion exchange chromatography the major part of the ototoxicity in crude Hib bacterial supernatant was fractionated to a sample containing 1.3 $mgml^{-1}$ of protein which is 4 % of the crude protein content (30.3 $mgml^{-1}$).

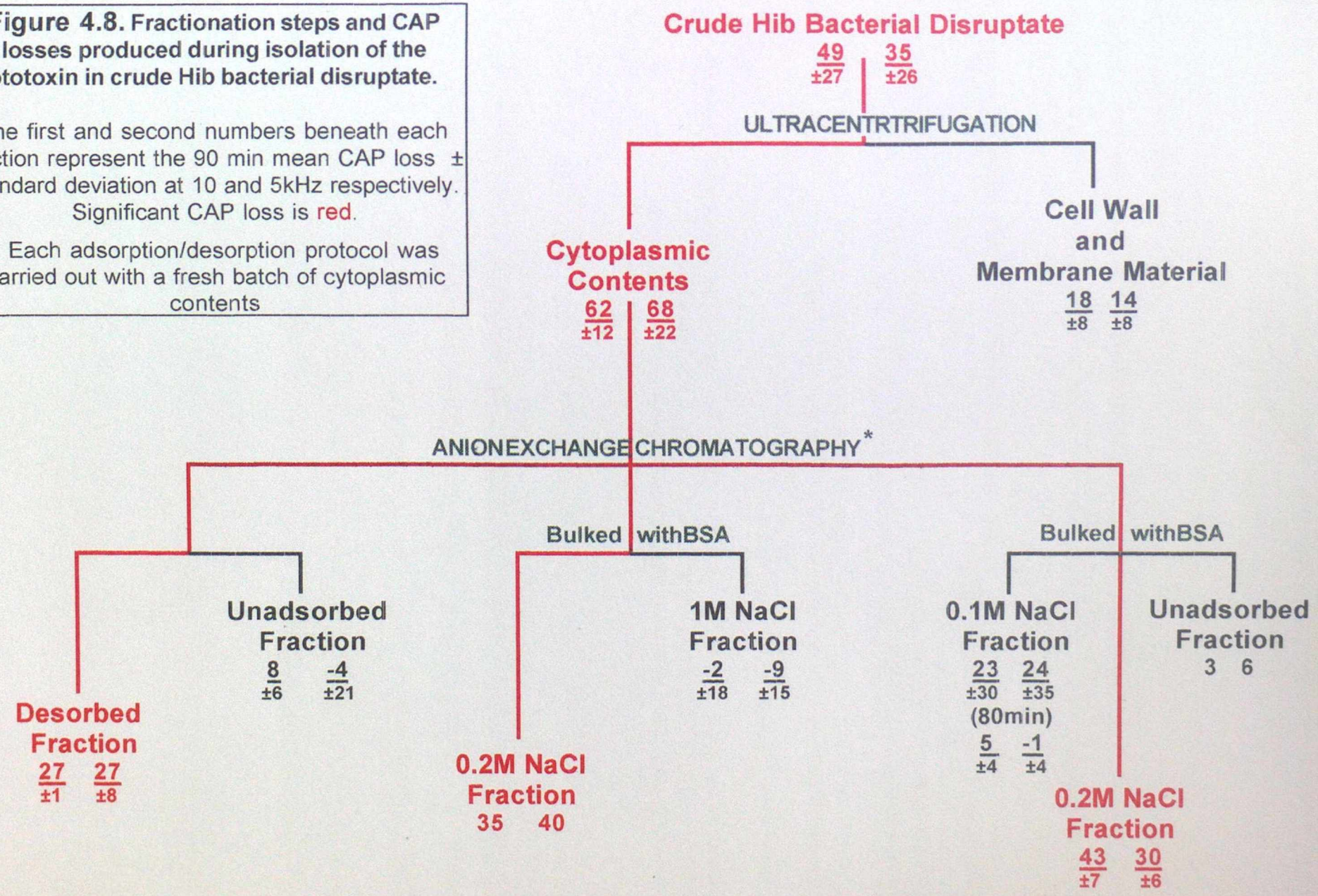
- The majority of the ototoxicity of the Hib extracts was found in a fraction which adsorbed to DEAE sepharose at pH 7.5 and eluted by 0.2M NaCl. This suggests that the ototoxic factor is negatively charged at pH 7.5.

- Bulking of the samples with BSA enhanced maintenance of ototoxic activity. This may be due to the presence of protease/s within the extracts which fractionate with the ototoxic factor. The result indicates that the ototoxin is a protein. However, this is not the only explanation for BSA-retention of ototoxicity. For example, BSA could have increased the viscosity of the sample which would limit oxygen transfer. If the ototoxic factor was inactivated by oxygen (e.g. it was thiol-activated) the activity of the sample could have been maintained simply by this physical effect on oxygen diffusion.

Figure 4.8. Fractionation steps and CAP losses produced during isolation of the ototoxin in crude Hib bacterial disruptate.

The first and second numbers beneath each fraction represent the 90 min mean CAP loss ± standard deviation at 10 and 5kHz respectively. Significant CAP loss is red.

* Each adsorption/desorption protocol was carried out with a fresh batch of cytoplasmic contents



4.9. Further Experiments

The results presented in this section were carried out using one batch of crude Hib bacterial disruptate received from CAMR. Subsequent batches of crude Hib bacterial disruptate yielded less reproducible results and displayed mild ototoxicity in comparison to the data presented here. Therefore it appears that the toxin is either labile in preparation or produced highly varying levels of ototoxicity. Future experiments would involve changes in methodology and *in vitro* conditions of culture; this is discussed in the Chapter 11 **(Section 11.2.3)**.

CHAPTER 5

Characteristics of 'Hib Ototoxin'

5.1. Electrophysiological Observations

In the majority of cases (1 exception), positive losses in CAPs involved an immediate large loss followed by a slow continued decline. 80-90% of the final loss in CAPs was achieved within 20 min, often within 5 or 10 min. Losses in CAPs were similar at the 10 and 5 kHz frequencies, but not at 3/1kHz. Mean 90 min CAP losses of all animals which displayed some ototoxicity (as judged by CAP loss >20dB) were 39, 38 and 22 dB at 10, 5 and 3/1 kHz respectively. Of these experiments, 48% had greatest CAP losses at 10kHz, 48% had greatest CAP losses at 5kHz, and the remainder (2%) had equal (greatest) CAP losses at 10 and 5kHz. 91% of these experiments displayed smallest CAP losses at 3kHz.

A positive loss in the CM was always accompanied by a positive loss in CAP, and in 75% of these the CAP loss was greater than 40dB. In contrast, only 17% of animals which displayed a positive loss in CAP at 90 min also displayed a positive loss in the CM at 90 min. The time course of this CM loss was variable but in most cases a gradual decline of the CM was seen throughout the 90 min. Losses in the CM were often preceded by an initial small increase in the CM.

An example of the characteristic effects of the Hib ototoxin was seen in animal 3 which was treated with crude Hib bacterial disruptate (**Fig. 5**). CAP losses increased with frequency, and exhibited an immediate large loss followed by a gradual decline. The loss in the CM was gradual and was preceded by a small initial increase in the size of the CM.

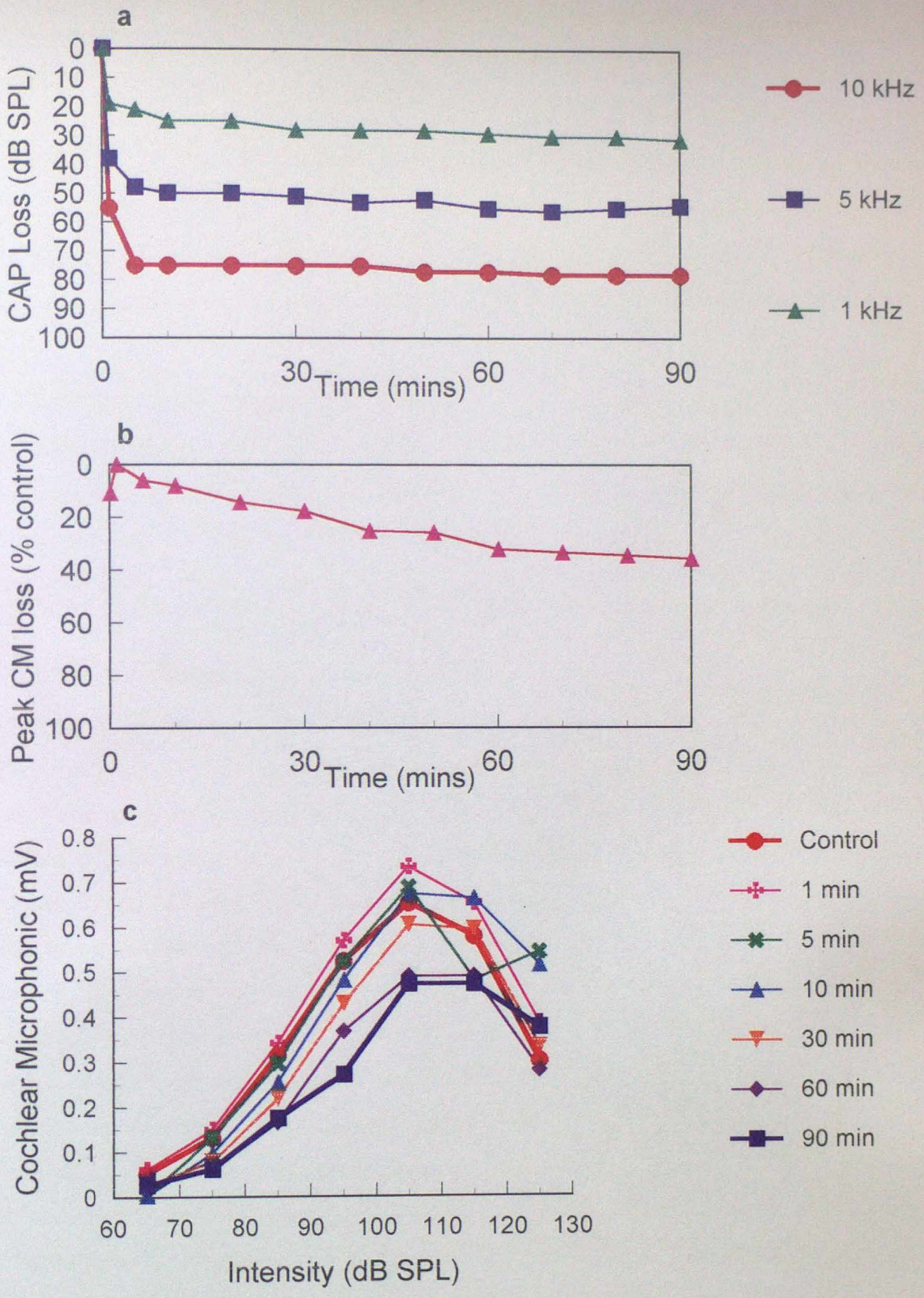


Figure 5. **CAP and CM losses produced by perfusion of crude Hib bacterial disruptate in animal 3.**

a) CAP losses at 10, 5 and 1 kHz b) Peak CM losses
c) CM losses at all stimulus intensities at 0, 1, 5, 10, 30, 60 and 90 min post perfusion.

5.2. Morphological Observations

Cochleae that displayed ototoxicity in terms of electrophysiological responses were examined by either transmission or scanning electron microscopy.

5.2.1. Scanning Electron Microscopy

Scanning electron microscopy of cochleae treated with crude 'Hib ototoxin' demonstrated scattered areas of damage along the organ of Corti. The damage was not consistent amongst treated cochleae. Damage observed included cratering in the supporting cells, and splaying of the stereocilia of the outer and inner hair cells particularly adjacent to sites of cratering (**Plates. 5 i-ii**).

5.2.2. Transmission electron microscopy.

All cochleae treated with crude 'Hib ototoxin' displayed similar damage when examined by TEM (**Plates 5 iii-vi**). The most striking damage observed was to the mitochondria. The mitochondria were swollen and in some cases the whole contents were missing. In some cells mitochondria were swollen to more than twice their original size as compared to control (untreated) cochleae. The greatest degree of mitochondrial damage was observed in afferent and efferent nerve endings to inner and outer hair cells. However mitochondrial damage was not specific to cell type, because swollen and empty mitochondria were also seen in outer and inner hair cells, in supporting cells, and also in the basilar membrane. Treatments which produced positive CM losses appeared to be associated with more extensive mitochondrial damage than treatments which did not produce CM losses. Another common feature amongst treated cochleae was membrane damage. Both cell membranes and organelle membranes were often thin, broken or absent. The cytoplasmic contents of the inner hair cells were often thin or missing in patches and there were frequent indications of cellular debris. In contrast, the cytoplasmic contents of the efferent nerve endings at the outer hair cells were often more dense than in control material.

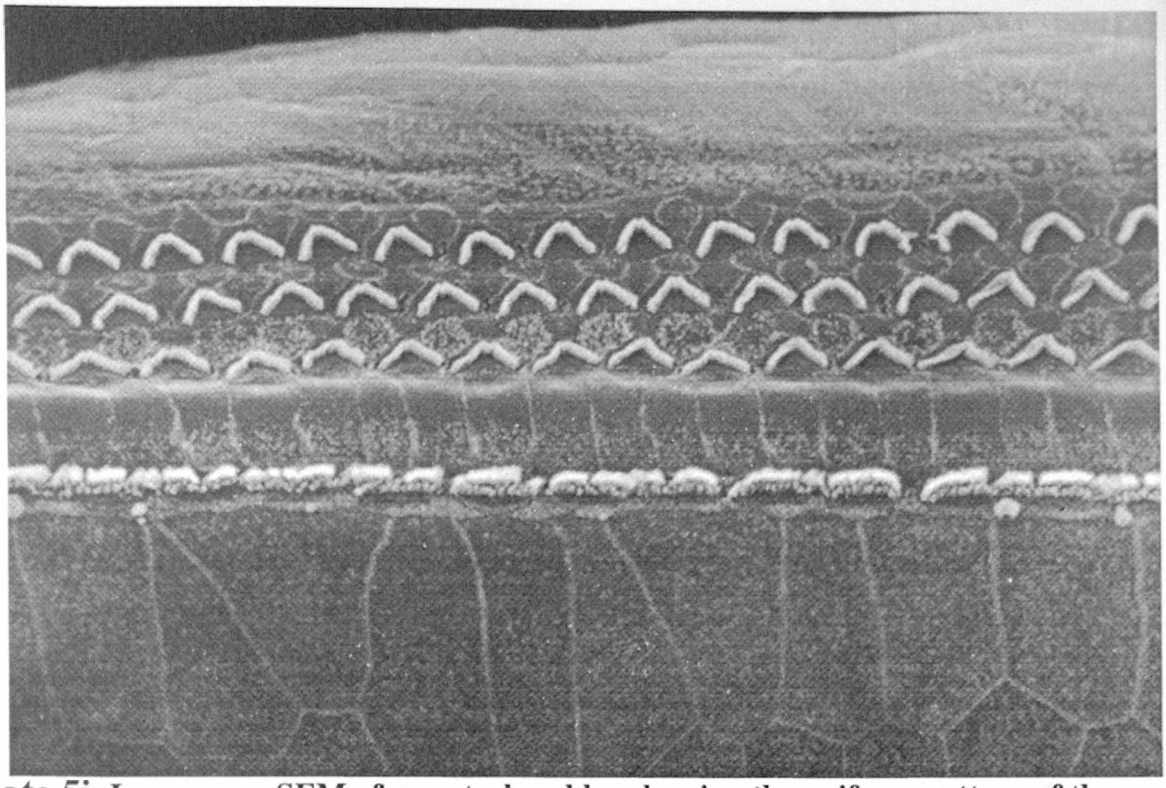


Plate 5i. Low power SEM of a control cochlea showing the uniform pattern of the organ of Corti with 3 rows of outer hair cells and 1 row of inner hair cells. Magnification x 1000.

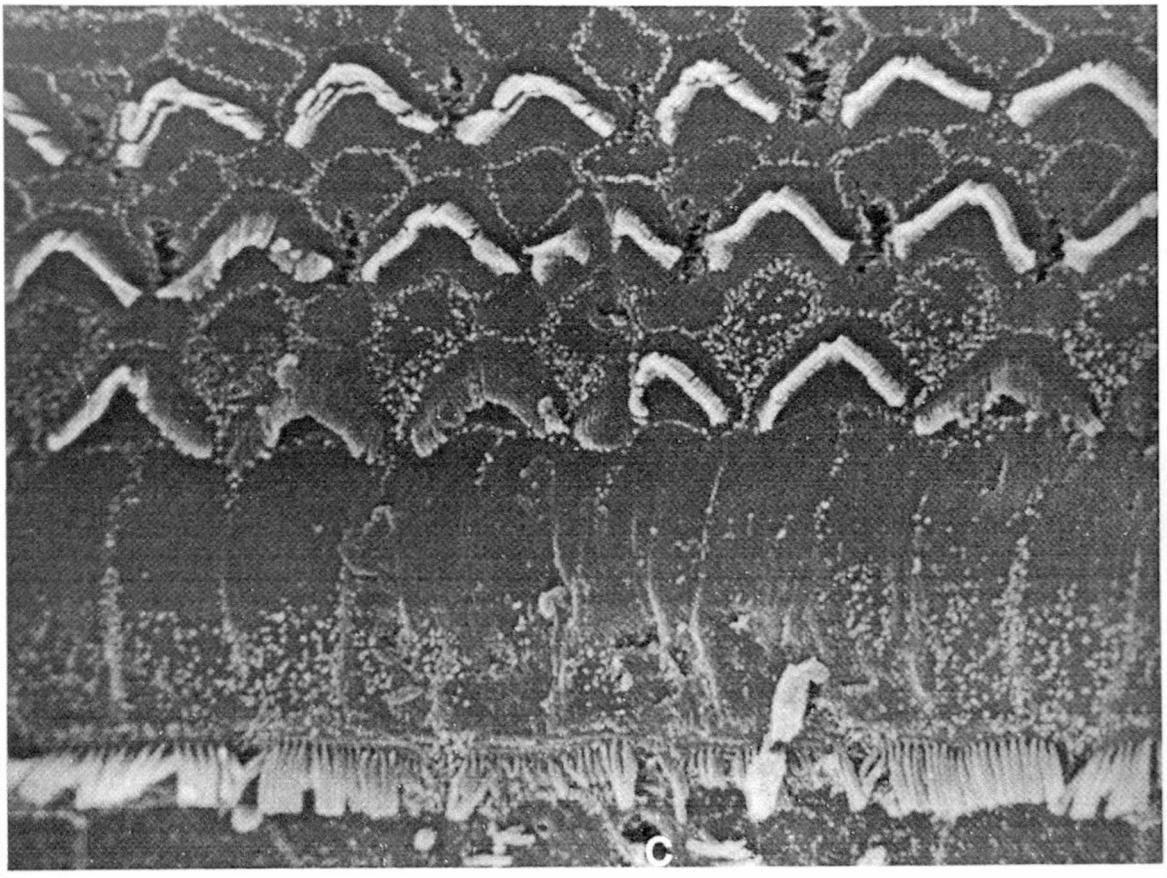


Plate 5ii. Low power SEM of a cochlea treated with cytoplasmic contents of the Hib bacterial extract. There is splaying and detachment of stereocilia of both the outer and inner hair cells. The stereocilial damage is co-located with damage to the apical surface of supporting cells in the form of a crater (C). Magnification x 2500

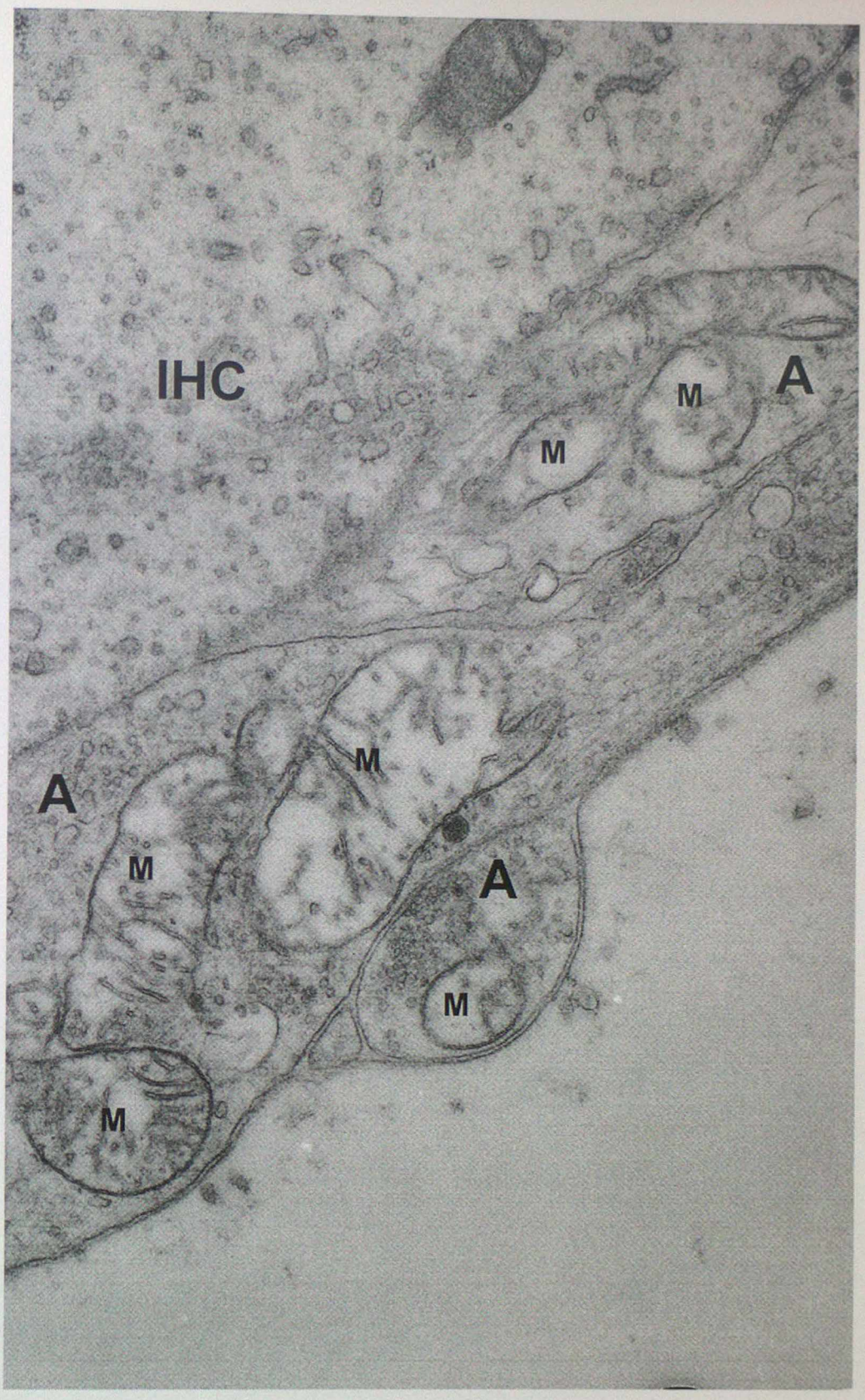


Plate 5iii TEM of the nerve endings at the base of an IHC of the left cochlea from an animal treated with cytoplasmic contents of the Hib bacterial extract. There is striking swelling of the mitochondria (M) in the afferent nerve endings (A) at the base of an IHC.
Magnification x 30000.

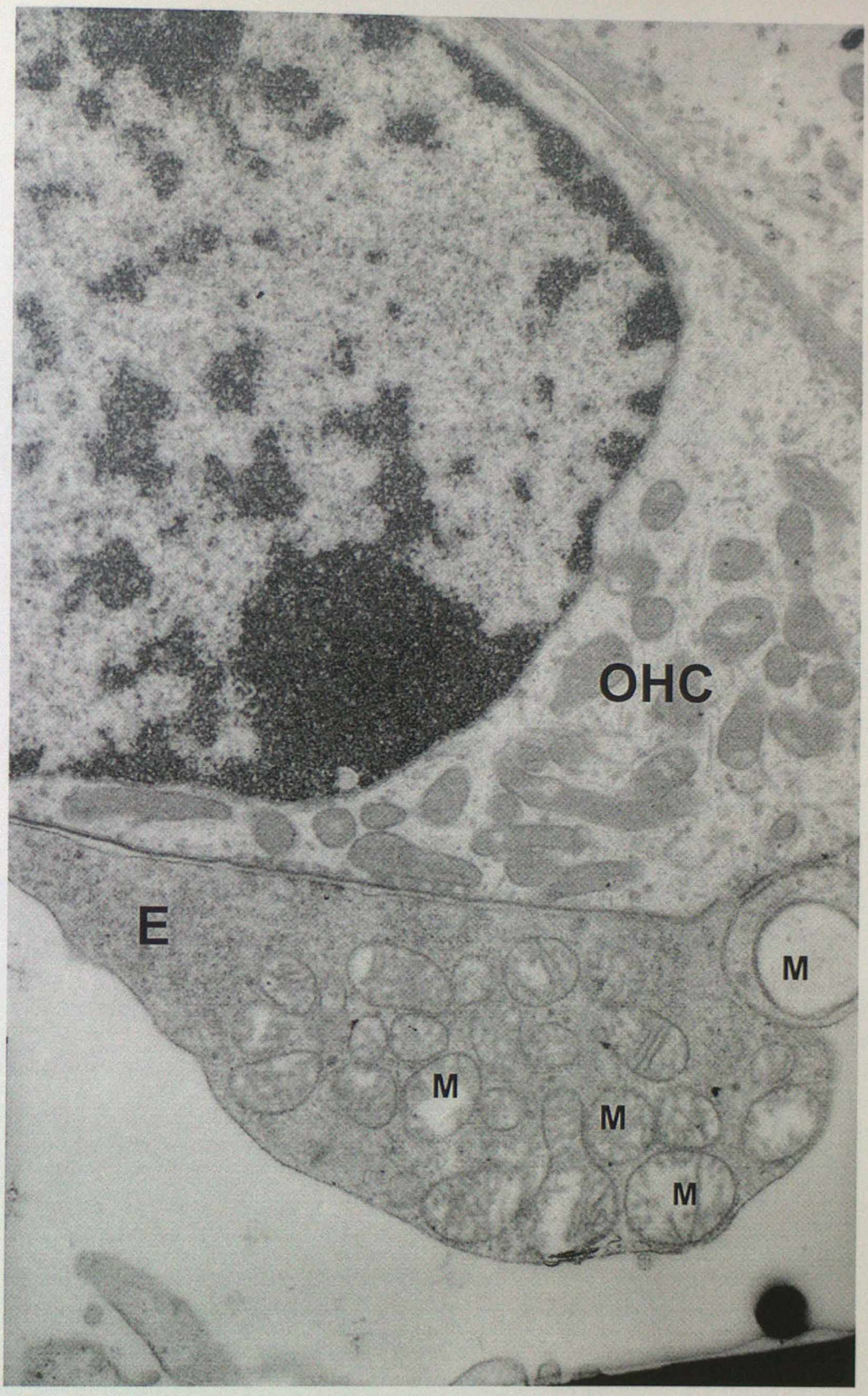


Plate 5iv TEM of a row 2 outer hair cell (OHC) of the left cochlea from an animal treated with cytoplasmic contents of the Hib bacterial extract. There is striking swelling of the mitochondria (M) in the efferent nerve ending (E) at the base of the OHC. Magnification x 21750.

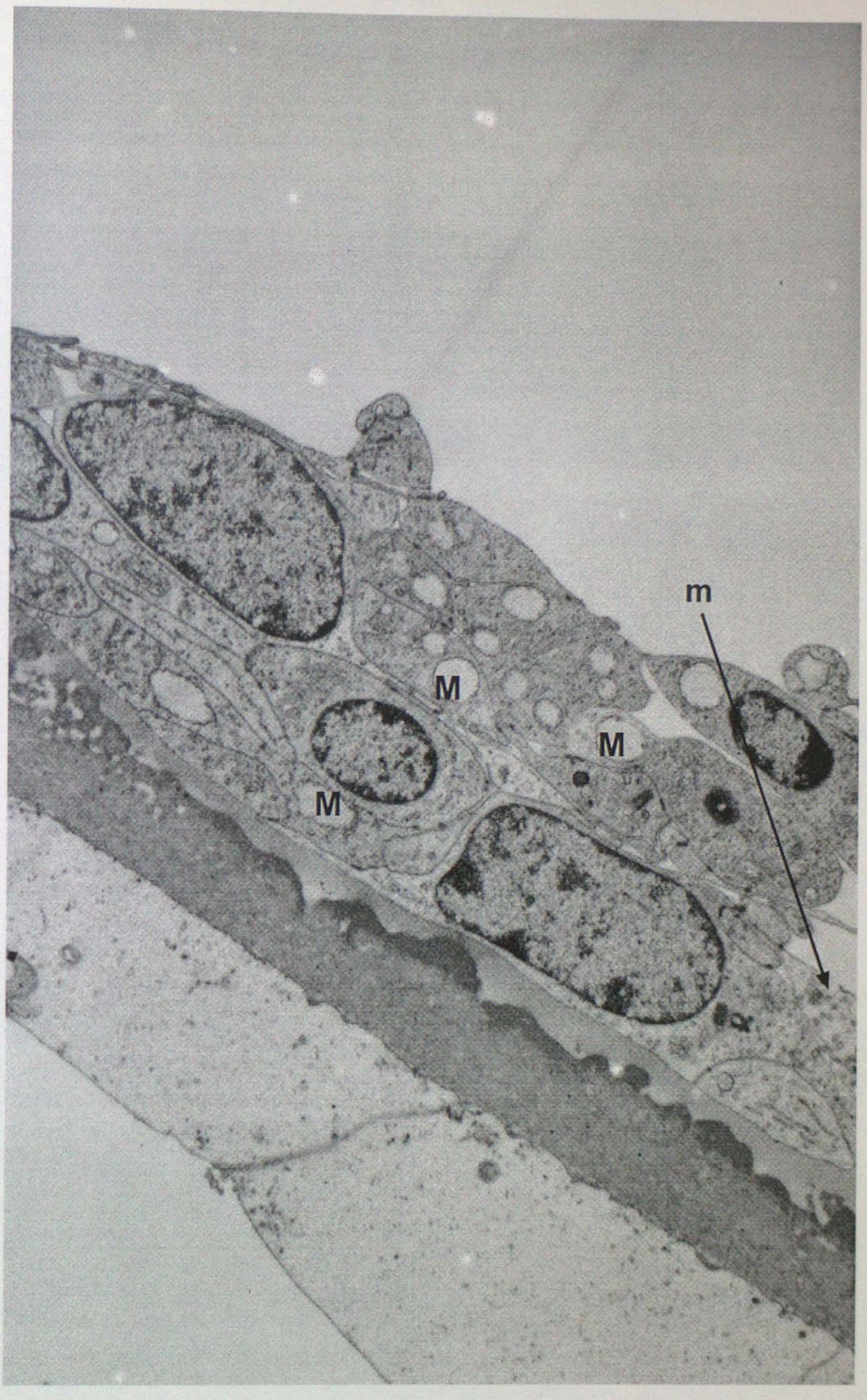


Plate 5v TEM of the basilar membrane of the left cochlea from an animal treated with cytoplasmic contents of the Hib bacterial extract. Swelling of the mitochondria (M) can be seen along with some signs of membrane damage (m). Magnification x 6300.

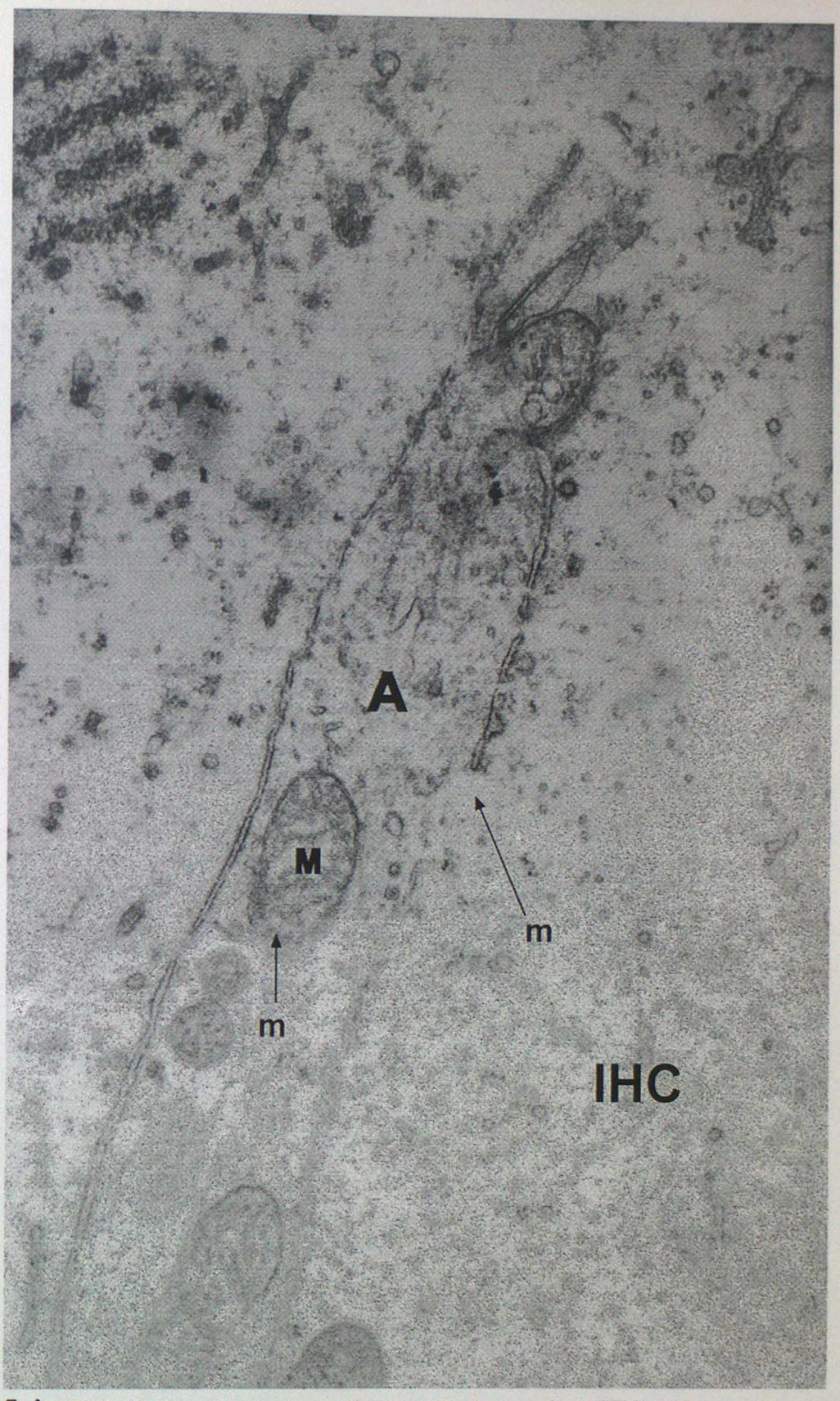


Plate 5vi TEM of afferent nerve endings at the base of an IHC of the left cochlea from an animal treated with cytoplasmic contents of the Hib bacterial extract. There is striking swelling of the mitochondria (M) in the afferent nerve ending (A) at the base of the IHC and associated membranous damage (m) of the nerve terminal and organnelles within it. Magnification x 30000.

5.3 Characteristics of 'Hib Ototoxin': Conclusions

- The 'Hib ototoxin' produced a consistent drop in CAPs which was sometimes accompanied by a fall in the CM, particularly when the fall in the CAP exceeded 40dB. This is likely to mean that the primary target of the ototoxic factor was either the afferent neurons, afferent synapse or inner hair cells (all of which affect the CAP). The inconsistency in CM loss suggests that the outer hair cells (main contributor to the CM) were a secondary target which were not always affected.

- CAP losses were similar at 10 and 5kHz, but not at 3kHz. Smaller CAP losses displayed at 3kHz may have been due to a lack of sensitivity to reduced activity in a small number of the fibres because of the greater number of contributing fibres to the CAP at this frequency. In addition smaller CAP losses displayed at 3kHz may have been a result poor penetration of the extract to the apex of the cochlea.

- The morphological evidence demonstrated consistent mitochondrial damage throughout the organ of Corti associated with the ototoxicity produced by Hib cytoplasmic fractions. Therefore it appears that the major target of the Hib ototoxin was mitochondria.

CHAPTER 6

Ototoxicity Of *N.meningitidis* type b Bacterial Extracts

Formal evidence for the role of pneumolysin in causation of deafness following pneumococcal meningitis has already been presented (Winter *et al.* 1997). Strongly suggestive evidence implicating a soluble toxin responsible for deafness associated with meningitis caused by Hib has also been presented here (section 4.0). In this section the question is addressed as to whether there are similar ototoxic proteins present in *N.meningitidis* functionally comparable to the ototoxic proteins in *S. pneumoniae* and *H.influenzae* type b. A virulent strain of *N.meningitidis* type b (of human clinical origin) was grown in bulk at CAMR, Porton Down. The culture was separated into culture filtrate and bacterial cells. As had been done with Hib, the organisms were disrupted at CAMR and all materials transported to Birmingham. To my knowledge the kind of experiments described in the following section have never been done before.

One large batch of meningococci was grown and worked up at CAMR. This was used for all the experiments described in this section. In total, 16 animals were used in the work described in this chapter, and the initial auditory thresholds can be seen in **Fig. 6**. Data from three animals were excluded from statistical and summary information as it was felt these animals had initial auditory thresholds outside the normal distribution in this group.

Aims And Objectives:

1. To test the ototoxicity of a crude meningococcal bacterial disruptate.
2. To fractionate the bacterial disruptate into a soluble fraction and pellet by ultracentrifugation.
3. To perfuse both crude and 'fractionated' meningococcal fractions into the scala tympani of guinea pig cochleae and monitor electrophysiological responses.
4. To examine, by electron microscopy, perfused cochleae if ototoxicity was observed in terms of loss of electrophysiological responses.

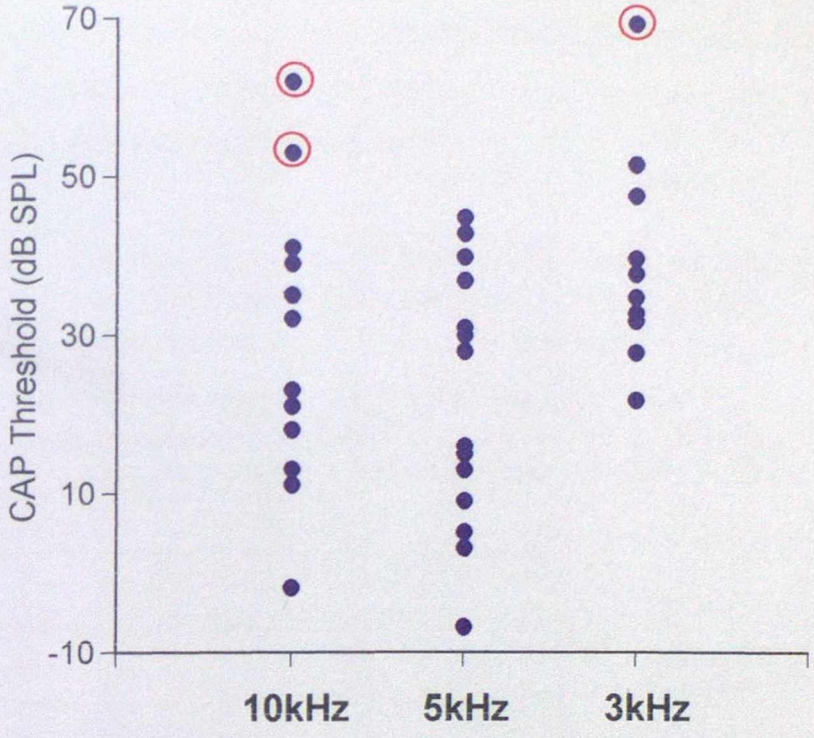


Figure 6. Threshold CAP values of all animals used in the work described in this chapter.

Red circles indicate thresholds either above the defined level
(50dB at 10 and 5kHz and 65dB at 3kHz),
or well outside the normal distribution of the group.

6.1 Crude Meningococcal Bacterial Disruptate

An initial experiment was carried out in which the crude meningococcal bacterial disruptate was perfused into the cochlea of one animal to give an indication of the ototoxicity of the crude extract (**Fig. 6.1**; **Table 6.1**). The crude bacterial disruptate produced no positive losses in CAPs or CMs. At 90 min, losses in the CAP were -5, -15 and 5dB at 10, 5 and 3 kHz respectively, and the loss in the CM was -4%.

Table 6.1 : Electrophysiological losses evoked by perfusion of a guinea pig cochlea with meningococcal bacterial disruptate. Positive losses are bold.

Time post perfusion (min)	Animal 1			
	CAP			CM
	10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) Peak CM (mV)			
	35	30	40	0.46
	Loss in CAPs (dB) or CM (% control)			
1	0	0	0	2
5	-3	-5	0	0
10	3	3	0	-2
30	-1	-3	0	4
60	-5 *	-17	3	4
90	-5	-15	5	-4

Conclusion

These initial data provided no evidence for an ototoxic factor in the crude meningococcal bacterial disruptate.

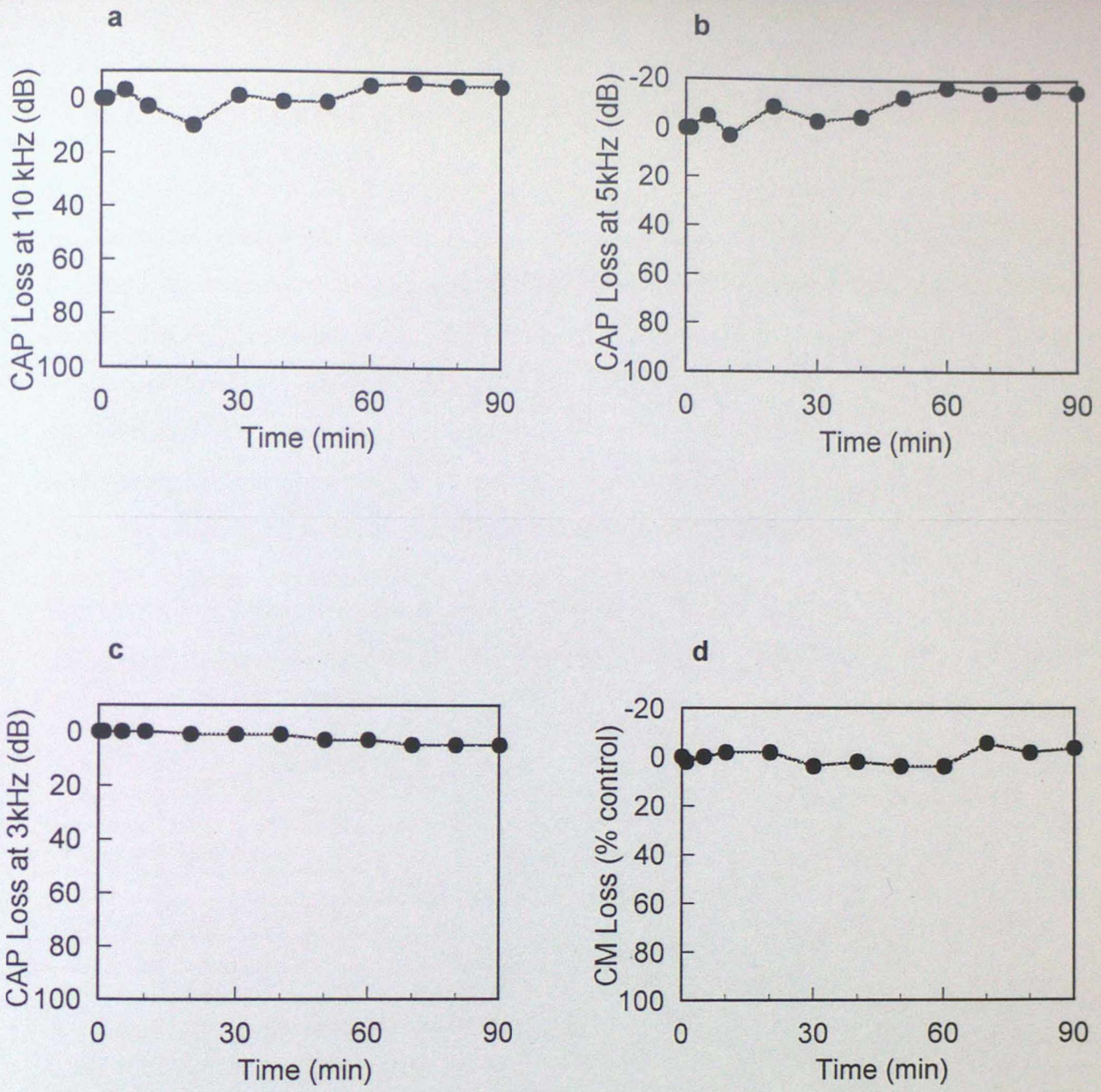


Figure 6.1. Losses in the CAP and CM in animal 1 treated with crude meningococcal bacterial disruptate .

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

6.2 Concentrated Crude Meningococcal Bacterial Disruptate.

As the initial experiment indicated no ototoxic activity in meningococcal crude bacterial disruptate, the extract was concentrated to $13mgml^{-1}$ and further assessed for ototoxicity in three animals (**Fig. 6.2**; **Table 6.2**). All animals perfused with this extract displayed 10kHz CAP losses in excess of 15dB at 1 min post perfusion. In all animals the CAP had fully recovered by 5 min post perfusion, probably due to a temperature effect (as discussed in Section 3.1). Evidence of CAP loss beyond the 1 min recording was seen in animals 2 and 3, but not in animal 4. At 90 min, loss in the 10kHz CAP was 27dB and 20dB in animals 2 and 3 respectively. No animal showed 5 or 3kHz CAP losses exceeding 20dB, or loss in CM exceeding 16%.

Table 6.2: Electrophysiological losses evoked by perfusion with concentrated crude meningococcal bacterial disruptate in three animals.

Positive losses are bold.

Time post perfusion (min)	Animal 2				Animal 3				Animal 4			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	18	37	52	0.51	21	13	33	0.27	32	45	-	0.33
	Loss in CAPs (dB) or CM (% control)											
1	17	20	-	4	39	17	8	8	16	3	-	3
5	10	20	-	6	12	9	4	6	0	0	-	0
10	11	13	-	4	15	14	9	-10	0	0	-	-2
30	**21**	11	-	7	**24**	13	10	-3	1	1	-	-3
60	**26**	11	-	12	**22**	13	7	0	0	0	-	8
90	**27**	11	-	14	**20**	10	6	-6	1	1	-	16

Conclusions

- There was *some* evidence for **transient** ototoxic activity produced by perfusion of crude concentrated meningococcal bacterial disruptate into the cochlea. However this may have been due to imperfect temperature equilibration of the sample after removal from the ice box.
- There was *some* evidence for **persistent** ototoxicity in crude concentrated meningococcal bacterial disruptate, however the results were not consistent between animals.

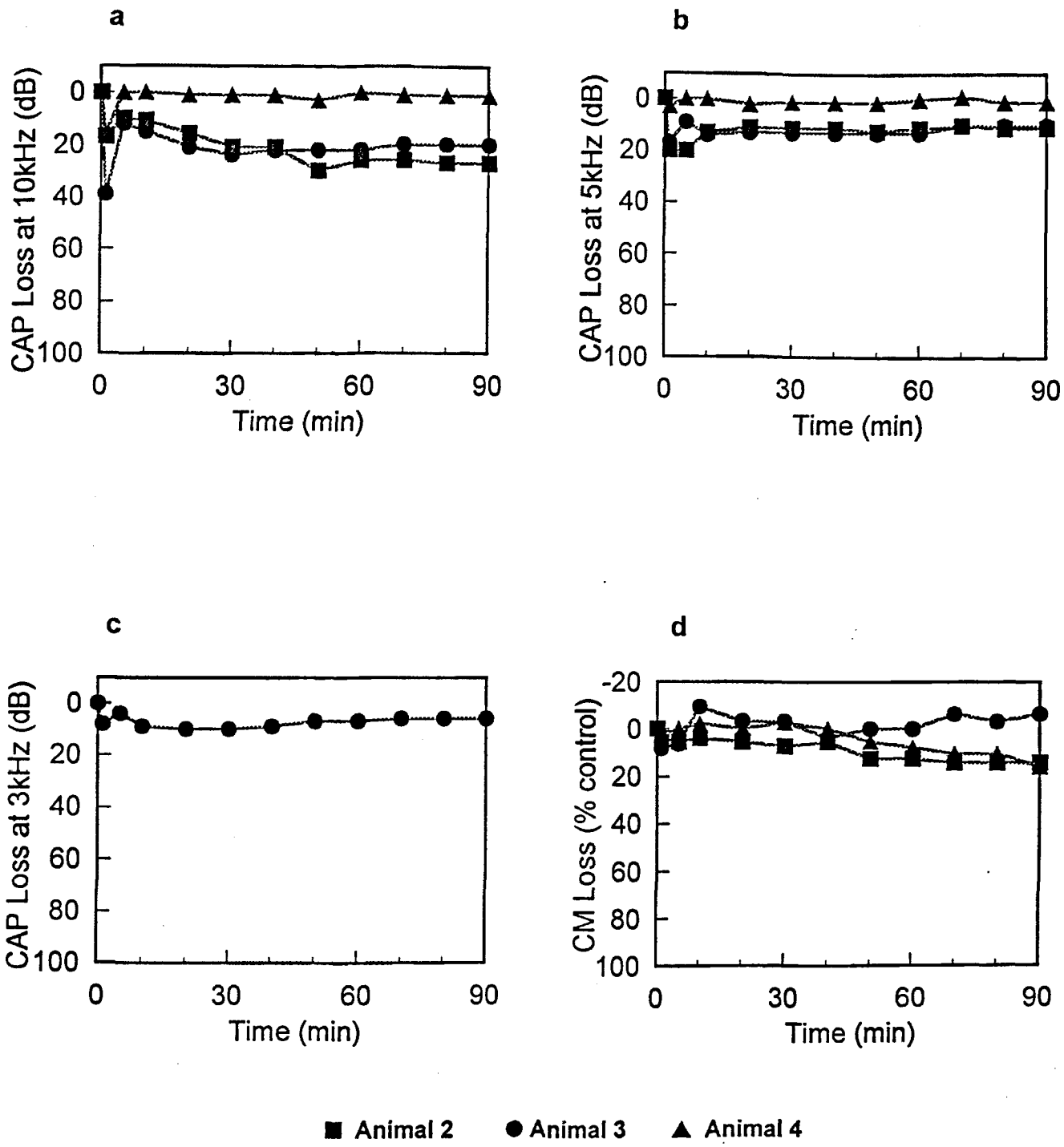


Figure 6.2. **Losses in the CAP and CM in three animals, each treated with concentrated crude meningococcal disruptate.**

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

- Since there was some evidence that concentration of the crude meningococcal bacterial disruptate resulted in increased ototoxicity, all further samples were concentrated to an equivalent level.

6.3 Dithiothreitol (DTT) Treatment Of Concentrated Crude Meningococcal Bacterial Disruptate.

Since crude pneumolysin is thiol-activated, it was proposed that a similar ototoxin produced by meningococci may also be thiol-activated. Crude pneumolysin is inactivated by SH oxidation. Hence the crude concentrated disruptate was treated with DTT, a reducing agent which would reduce functional SH groups.

6.3.1 DTT-Treated Bovine Serum Albumin (BSA).

It was considered worthwhile to test first for any possible non-specific effects of thiol reduction using BSA; BSA contains numerous SH groups (Swissprot) and hence is a good protein to test for the non-specific effects of -S-S- reduction. DTT-treated BSA was tested for ototoxicity in three guinea pigs (**Fig. 6.3.1**; **Table 6.3.1**). Animal 6 displayed no positive losses in CAP or CM. Animals 5 and 7 exhibited an initial loss in the 10kHz CAP in excess of 30 dB at 1 min post perfusion. Both animals showed some recovery by 10 min, probably due to a temperature effect (as discussed in Section 3.1.). Animal 5 showed no further losses; animal 7 did not recover fully, exhibiting losses in CAP of 16dB, 22dB and 15 dB at 10, 5 and 3 kHz respectively at the 90 min time point. In all animals there were no positive losses in the CM.

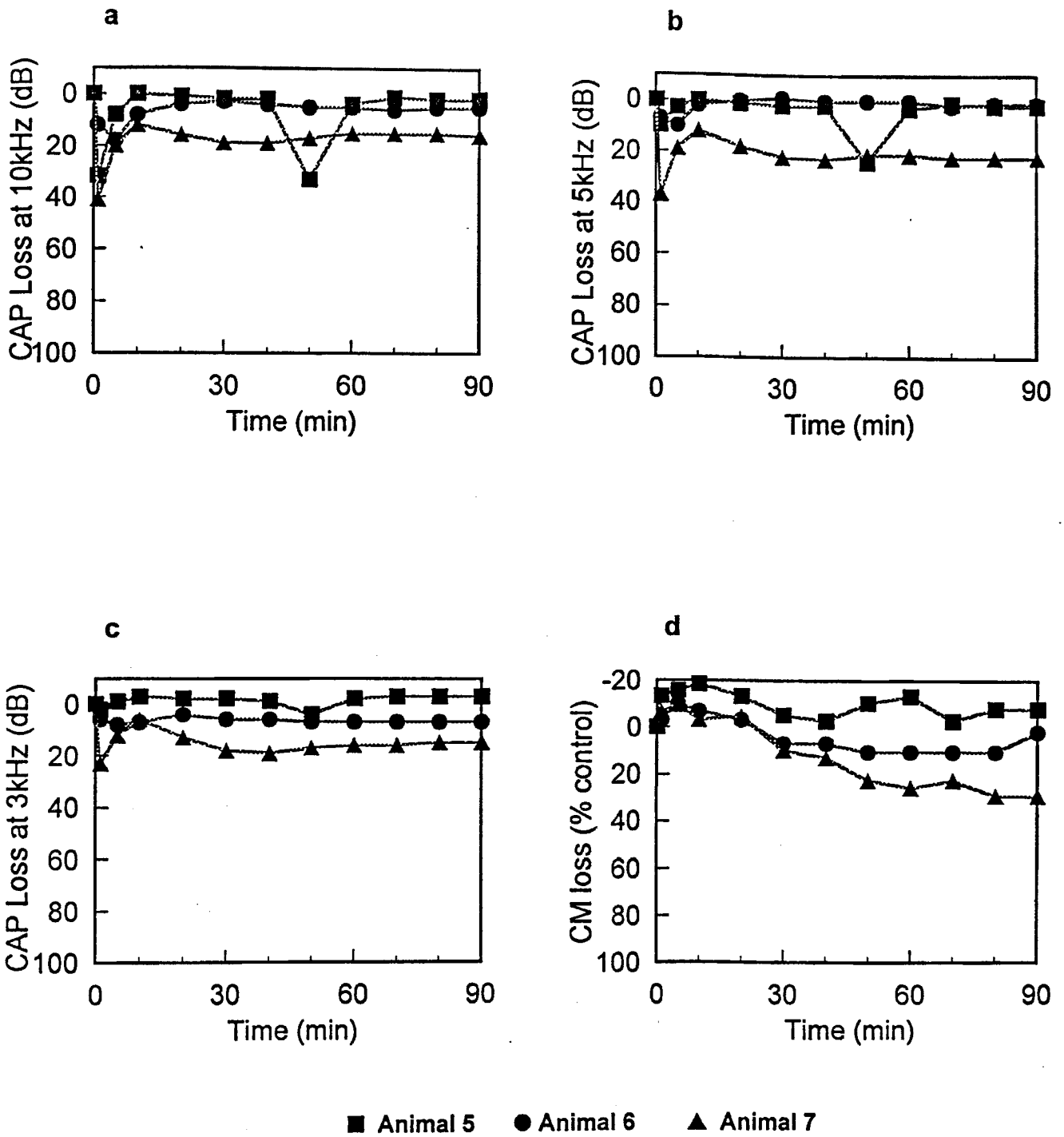


Figure 6.3.1. Losses in the CAP and CM in three animals, each treated with DTT-treated BSA.

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 6.3.1. Electrophysiological losses evoked by perfusion with DTT-treated BSA in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 5				Animal 6				Animal 7			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	23	28	48	0.33	41	16	38	0.25	11	3	32	0.27
	Loss in CAPs (dB) or CM (% control)											
1	**32**	10	2	-13	12	7	6	-3.	**41**	**37**	**23**	-6
5	8	3	-1	-16	18	10	8	-10	**20**	19	12	-10
10	0	0	-3	-18.	8	2	7	-7	12	12	6	-3
30	2	2	-2	-5	3	-1	6	7	19	**22**	18	10
60	4	3	-2	-13	5	0	7	10	15	**21**	16	26
90	2	2	-3	-8	5	1	7	2	16	**22**	15	29

Conclusions

- There was some evidence for *transient* ototoxicity produced by perfusion of DTT-treated BSA into the cochlea, however this may have been due to incomplete equilibration of the sample with ambient temperature.
- There was **some** tentative evidence for *sustained* ototoxicity in DTT-treated BSA, however, this was seen in only one animal and was positive at only one frequency. It may be that sustained ototoxic activity in meningococcal samples treated with DTT can be attributed to an SH-activated toxin. However, the data presented here implies that such data should be interpreted with some caution.

6.3.2 Concentrated DTT-Treated Meningococcal Bacterial Disruptate.

DTT-treated concentrated (13 $mgml^{-1}$) meningococcal bacterial disruptate was tested for ototoxicity in three animals (**Fig. 6.3.2; Table 6.3.2**). Data from animal 10 are included in Fig. 6.3.2 and Table 6.3.2a but have been excluded from statistical calculations as the animal displayed initial auditory thresholds outside the defined range. In animal 8 perfusion of DTT-treated concentrated bacterial disruptate produced an initial fall in the 10kHz CAP of 21 dB at 1 min, but this recovered by 5 min indicating a temperature effect. There were no further losses in the CAP

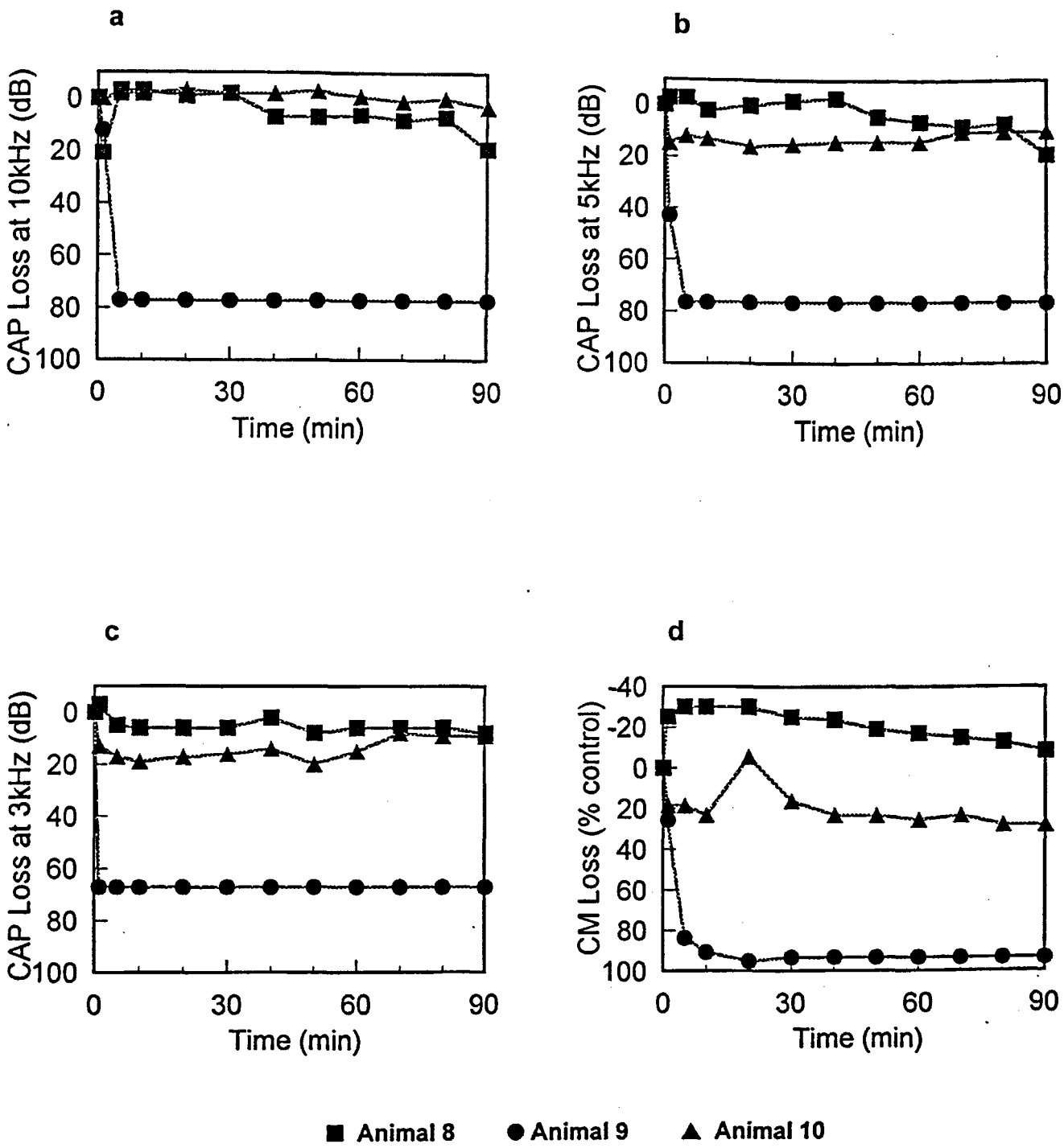


Figure 6.3.2. **Losses in the CAP and CM in three animals, each treated with concentrated DTT-treated crude meningococcal disruptate.**

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

or CM until 90 min, when 10 and 5kHz CAP losses reached 19dB which is just below the significance level. In contrast, animal 9 displayed losses in CAP in excess of 60dB at all frequencies, and a loss in CM of 93%. All detectable CAP was eliminated at all frequencies. Animal 10 had initial auditory thresholds of 33, 59 and 72 dB attenuation for the 10, 5 and 3kHz CAPs respectively of which only the 10kHz threshold is outside the defined range. In animal 10, perfusion of concentrated DTT-treated meningococcal bacterial disruptate produced no positive losses in the CAPs or CM. At 90 min, losses in the CAP did not exceed 10dB at any frequency, and the loss in the CM was 28%.

Table 6.3.2 : Electrophysiological losses evoked by perfusion with the concentrated and DTT-treated bacterial disruptate of *meningococcus* in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 8				Animal 9				Animal 10			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	-2	-7	28	0.41	18	13	37	0.38	62	31	33	0.38
	Loss in CAPs (dB) or CM (% control)											
1	**21**	-3	-3	-25	12	**43**	**67**	26	0	15	13	19
5	-3	-3	5	-30	**77**	**76**	**67**	**84**	-2	12	17	19
10	-3	2	6	-30	**77**	**76**	**67**	**91**	-2	13	19	23
30	-2	-2	6	-25	**77**	**76**	**67**	**93**	-2	15	16	16
60	6	6	6	-17	**77**	**76**	**67**	**93**	-1	14	15	26
90	19	19	8	-9	**77**	**76**	**67**	**93**	3	10	9	28

Conclusions.

- Data from animal 9 indicated the presence of a potent ototoxic factor, however this was not supported by the data from animals 8 and 10.
- Overall, there was some evidence for ototoxic activity in DTT-treated crude bacterial meningococcal disruptate. However the data were not consistent between animals.
- DTT treatment did not consistently increase the ototoxic activity of the crude concentrated meningococcal bacterial disruptate. Therefore DTT treatment was not routinely carried out.

6.4. Ultracentrifugation Of Crude Meningococcal Bacterial Disruptate.

The crude meningococcal bacterial disruptate contained cell wall, cell membrane and cytoplasmic components of meningococci. It is possible that any ototoxin in this crude extract may be inactivated or neutralised by the presence of other component/s in a manner that would not be the case *in vivo*. For this reason an initial ultracentrifugal step was carried out in an attempt to separate the soluble fraction from cell membrane and cell wall material. Both the supernatant fraction and resuspended pellet were assessed for ototoxicity.

6.4.1 Supernatant Fraction (Cytoplasmic Contents) Of The Concentrated Meningococcal Bacterial Disruptate.

The supernatant fraction was tested for ototoxicity in three animals (**Fig. 6.4.1** and **Table 6.4.1**). The data from animal 11 are included in Fig. 6.4.1 and Table 6.4.1 a, but have been excluded from statistical analysis because the animal displayed initial auditory thresholds outside the defined range. In all animals perfused with the supernatant fraction no positive losses in CAP or CM were observed. At 90 min post perfusion losses in CAP did not exceed 5dB, and the loss in CM did not exceed 20%.

Table 6.4.1 : Electrophysiological losses evoked by perfusion with meningococcal cytoplasmic contents in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 11				Animal 12				Animal 13			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	62	43	70	0.24	13	3	35	0.53	18	5	22	0.46
	Loss in CAPs (dB) or CM (% control)											
1	2	-1	-1	11	1	2	2	8	4	3	1	0
5	-9	-7	-4	7	1	6	3	8	5	6	4	0
10	-6	-3	-1	11	3	10	8	17	5	6	8	-2
30	-1	1	1	15	3	10	3	13	5	8	7	-4
60	3	5	1	15	0	7	3	23	5	8	6	-4
90	4	5	-2	15	-1	-2	0	20	1	5	5	4

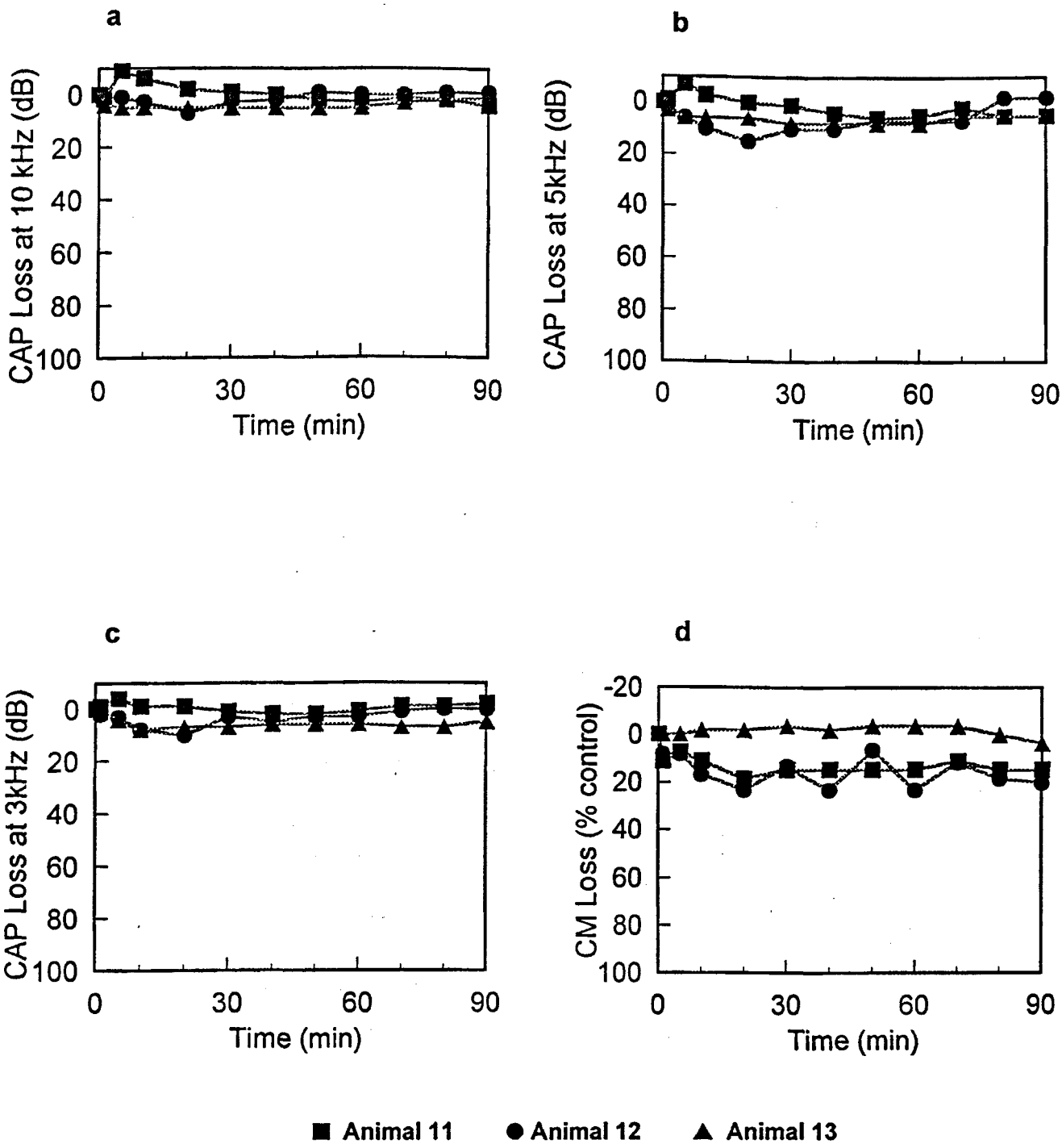


Figure 6.4.1. Losses in the CAP and CM in three animals, each treated with concentrated meningococcal cytoplasmic contents.

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

6.4.2 Pellet Fraction (Cell Wall and Membrane Material) Of The Concentrated Crude Meningococcal Bacterial Disruptate.

The pellet fraction of the concentrated meningococcal bacterial disruptate was resuspended in APL and tested for ototoxicity in three animals **(Fig. 6.4.2 ; Table 6.4.2)**. Data from animal 15 are included in Fig. 6.4.2 and Table 6.4.2 a, but have been excluded from statistical calculations as the animal displayed initial auditory thresholds above the defined level. In all animals perfused with the pellet fraction no positive losses in CAP or CM were observed. At 90 min post perfusion losses in the CAP did not exceed 6dB, and the loss in CM did not exceed 20%.

Table 6.4.2 : Electrophysiological losses evoked by perfusion with the cell wall and membrane material of the concentrated meningococcal bacterial disruptate.
Positive losses are bold.

Time post perfusion (min)	Animal 14				Animal 15				Animal 16			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	39	13	35	0.35	53	40	48	0.30	18	9	35	0.51
	Loss in CAPs (dB) or CM (% control)											
1	10	10	5	12	2	2	-2	-3	12	11	5	0
5	5	8	3	10	-2	-5	-2	0	6	5	4	4
10	5	8	3	10	-5	-3	-2	-6	4	4	2	-1
30	-1	2	0	5	-8	-7	-4	-3	5	5	2	2
60	0	4	2	17	-3	-5	-1	7	5	3	0	-1
90	1	6	4	20	-2	-4	-1	12	0	2	-1	-1

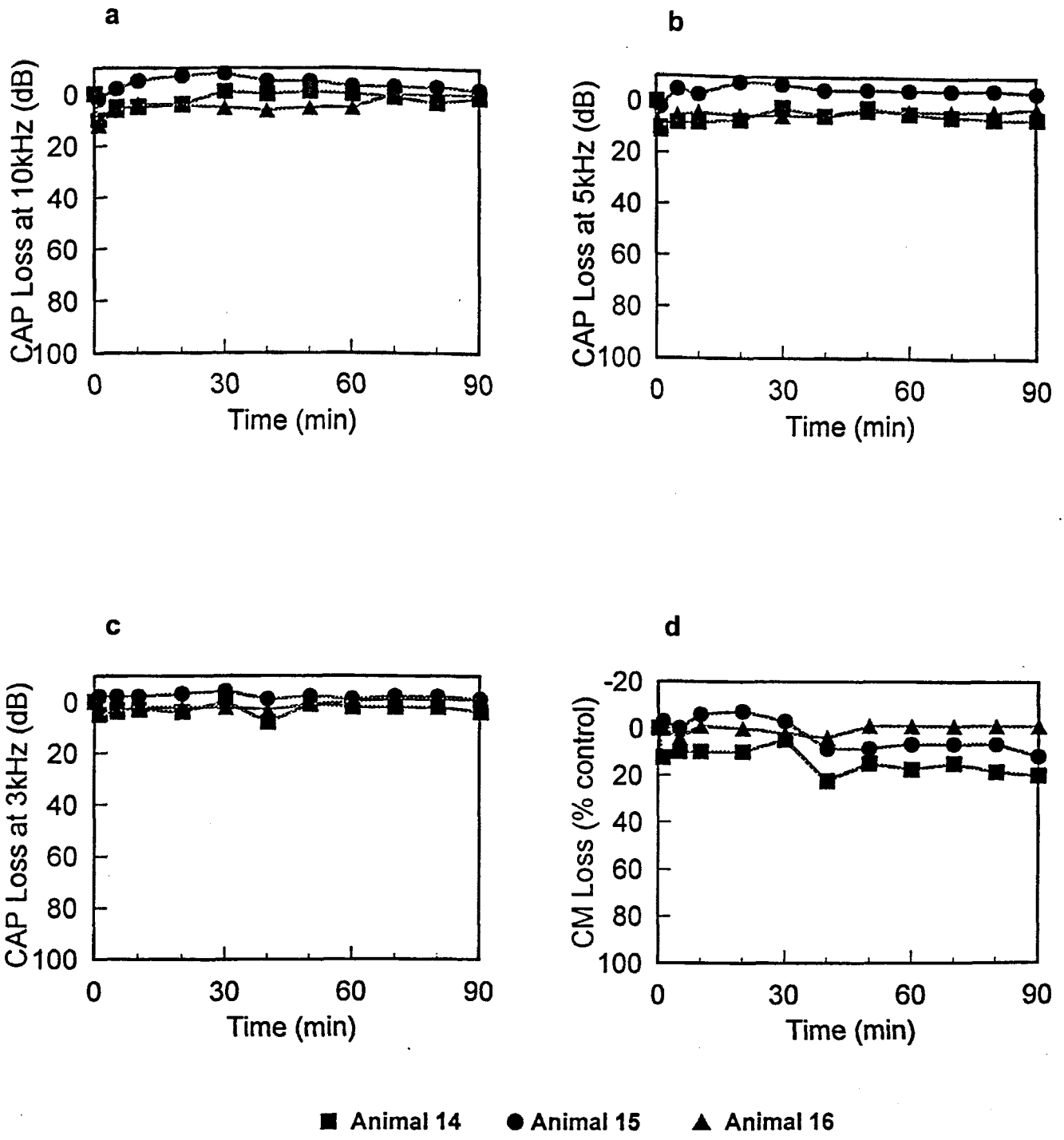


Figure 6.4.2. Losses in the CAP and CM in three animals, each treated with concentrated meningococcal cell wall and membrane material.

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

6.4.3. Ultracentrifugation of Concentrated Crude Meningococcal Bacterial Disruptate : Conclusions

- There was no evidence of ototoxicity in either the cell wall and membranous material or the cytoplasmic material.
- Fractionation of the crude bacterial extract by ultracentrifugation did not increase the ototoxicity of the extract. This would suggest that there is no particulate inhibitor present in the crude extract which might be inhibiting the action of a soluble toxin.
- The resuspended pellet fraction would have contained considerable amounts of LOS. The data presented here suggests that LOS within the crude meningococcal bacterial disruptate is not directly ototoxic in the guinea pig.

6.5. Summary Of The Meningococcal Bacterial Disruptate Cochlear Perfusions.

6.5.1 Statistical Analysis

A summary of the mean (± standard deviations) CAP and CM losses from all the experiments carried out on this meningococcal preparation is given in **Fig. 6.5.1** and **Table 6.5.1 a.**

Table 6.5.1. a: Average losses in the CAP and CM evoked by each treatment group at 90 min post perfusion. Positive losses are bold.

	CAP			CM
	10kHz	5 kHz	3kHz	
Bacterial Extract	Average loss in CAPs (dB) or CMs (%) at 90 min			
Crude (n=1)	-5	-15	5	-4
Crude Concentrated (n=3)	16 ± 13	7 ± 6	6	8 ± 12
DTT treated BSA (n=3)	8 ± 7	8 ± 2	6 ± 9	8 ± 19
DTT+concentrated crude (n=2)	**33 ± 39**	**35 ± 36**	**28 ± 34**	**38 ± 51**
Cytoplasmic Fraction (n=2)	0 ± 1	1 ± 5	2 ± 4	12 ± 11
Pellet Fraction (n=3)	0 ± 2	1 ± 5	1 ± 3	10 ± 11

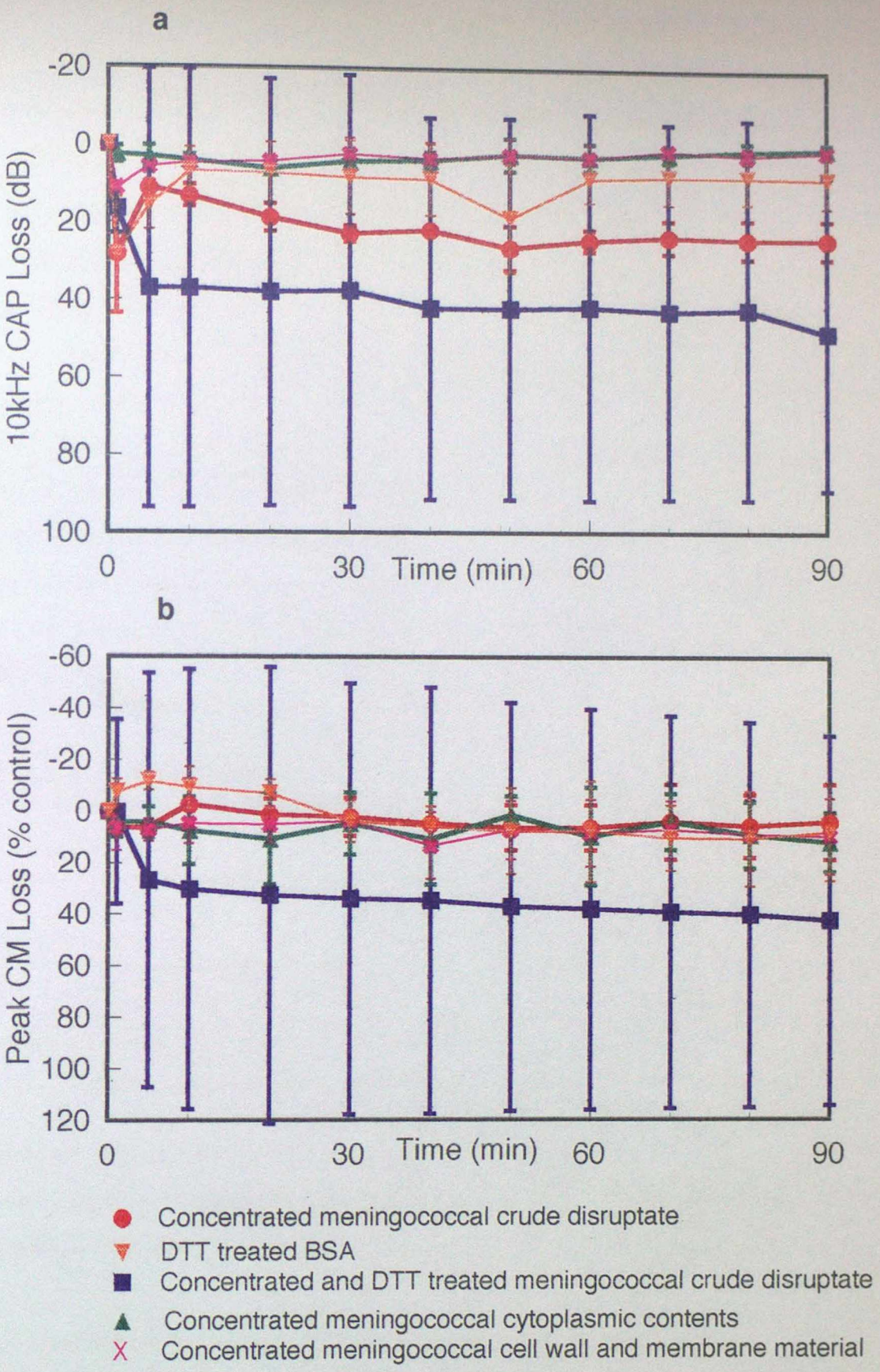


Figure 6.5.1. Average losses (±SDs) in the CAP and CM produced by five different treatments.

a) Losses in the CAP elicited with a 10kHz stimulus. b) Losses in the CM elicited with a 5kHz stimulus.

Statistical analysis of 10kHz CAP losses by ANOVA demonstrated no statistical significance between all treatments groups with respect to treatment alone (**Table 6.5.1 b**). However, there were significant differences between some treatment groups with respect to the change in the CAP with time. There was a statistical significance between the DTT-treated BSA data and meningococcal cytoplasmic contents data with respect to the change in the CAP with time ($p=0.006$). However, closer inspection of the results indicates that this represents a difference in the 10kHz CAP losses at 1 and 5 min only which is likely to be due to differential cooling effects on the cochlea rather than differential ototoxicity produced by the extracts. A significant difference was also calculated between concentrated meningococcal extract data and pellet fraction data in terms of the change in the CAP with time ($p=0.02$). This indicates some statistical significance between the ototoxicity produced by concentrated meningococcal extract and the pellet fraction.

Table 6.5.1. b: Statistical results (P values) of ANOVA analysis between each treatment with respect to the change in the CAP with time.
Significant differences are bold.

	DTT/BSA	Conc Crude	DTT+Conc Crude	Supernatant Fraction
DTT/BSA				
Conc Crude	0.3			
DTT+Conc Crude	0.3	0.8		
Supernatant Fraction	**0.006**	0.05	0.7	
Pellet Fraction	0.08	**0.02**	0.4	0.2

The whole set of data can be seen more clearly in **Fig. 6.5.2**. Statistically, the data produced by perfusion of all bacterial extracts are not significantly different to the data from perfusion of DTT-treated BSA.

6.5.2. Conclusions

- The variability in animal responses to these preparations could be interpreted to mean that there is no meningococcal ototoxin.

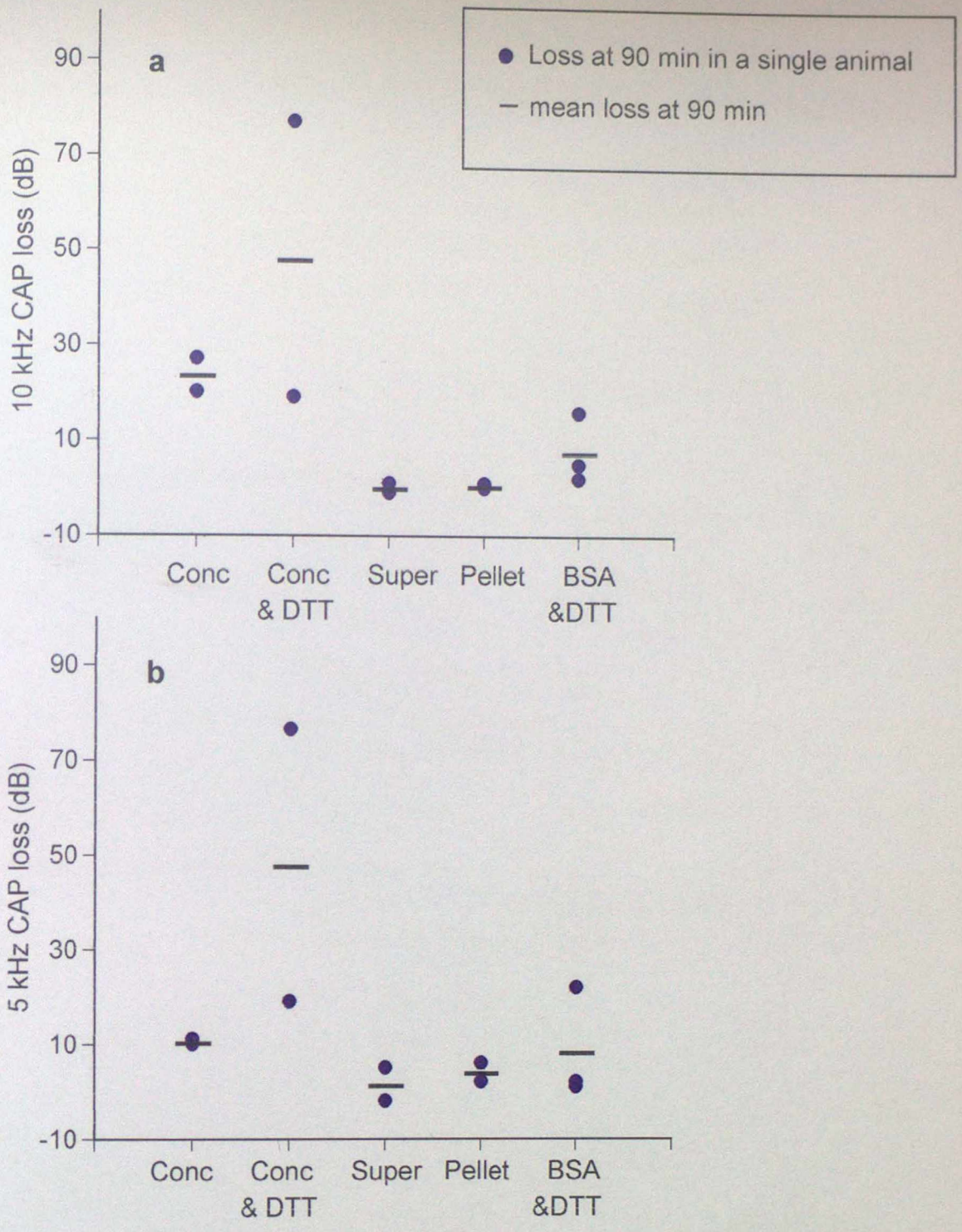


Figure 6.5.2. Losses to the auditory potentials at 90 min post perfusion, produced by 5 treatment groups.

a-b) CAP elicited with 10, 5kHz stimuli respectively.
Treatment groups were : Concentrated Crude Meningococcal Bacterial Extract (**Conc**), DTT treated 'Conc' (**Conc & DTT**), Supernatant fraction of 'Conc' (**Super**), Pellet fraction of 'Conc' (**Pellet**) and DTT treated BSA (**BSA & DTT**).

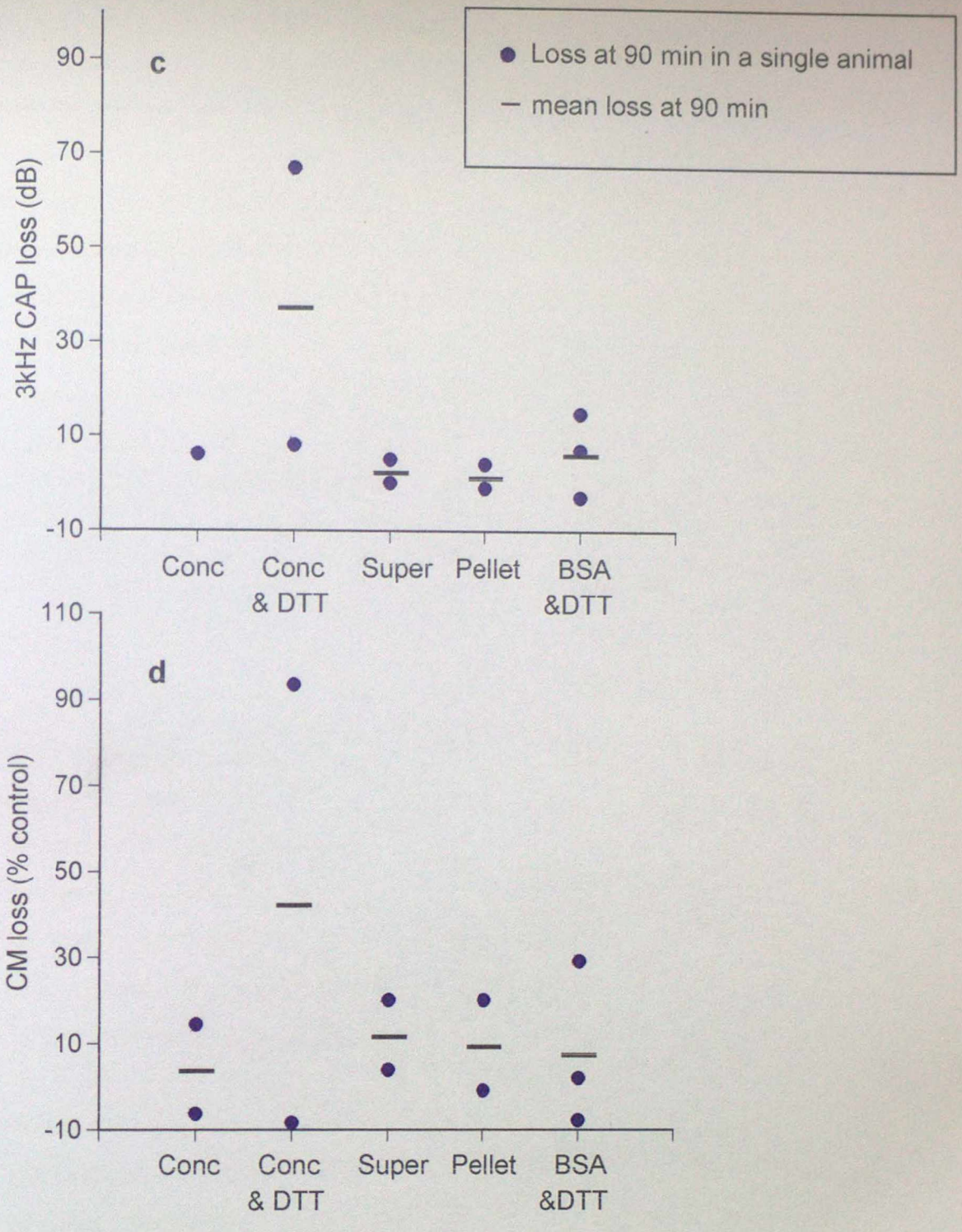


Figure 6.5.2. Continued **Losses to the auditory potentials at 90 min post perfusion, produced by 5 treatment groups.**

c) CAP elicited with 3kHz stimuli d) CM elicited with 5kHz stimuli.
Treatment groups were : Concentrated Crude Meningococcal Bacterial Extract (**Conc**), DTT treated 'Conc' (**Conc & DTT**), Supernatant fraction of 'Conc' (**Super**), Pellet fraction of 'Conc' (**Pellet**) and DTT treated BSA (**BSA & DTT**).

- Alternatively, one might conclude that there was a meningococcal ototoxin and that the inconsistency of the ototoxic data was an expression of the variation in sensitivity of the animals to low concentrations of that ototoxin. In the next section (6.6) an attempt was made to detect any 'pneumolysin-like' protein(s) present in the meningococcal extracts by using monoclonal antibodies to pneumolysin with which to probe SDS-PAGE gel analyses of the meningococcal extracts.

6.6 Protein Analysis

The crude meningococcal bacterial extract was analysed by SDS polyacrylamide-gel electrophoresis and probed with specific polyclonal anti-pneumolysin antisera to determine whether there were any 'pneumolysin-like' proteins within meningococcus (**Fig. 6.6**). This proceedure was carried out twice and produced similar results on both occasions. The major band within pneumococcal extracts which is recognised by the anti-pneumolysin antisera runs at around 53kDa. There is a band (of less intensity) of comparable size present in crude Hib bacterial disruptate. There is also a band (of less intensity than pneumococcal and Hib bands) present in the meningococcal extract of comparable size together with a smaller band (ca. 32kDa).

Conclusions

- Hib bacterial disruptate contains a protein with similar RF to pneumolysin, but there is far less of it than pneumolysin.
- There is a protein present in the meningococcal extract which has the same Rf as pneumolysin and the Hib protein (recognised by the anti-pneumolysin antisera) which could be a pneumolysin-like ototoxin. There is clearly less of this protein present than in Hib, and certainly much less than in pneumococcus.
- There is also a smaller protein present (ca. 32KDa) in the meningococcal extract which may be an inactive degradation product of the pneumolysin-like protein.

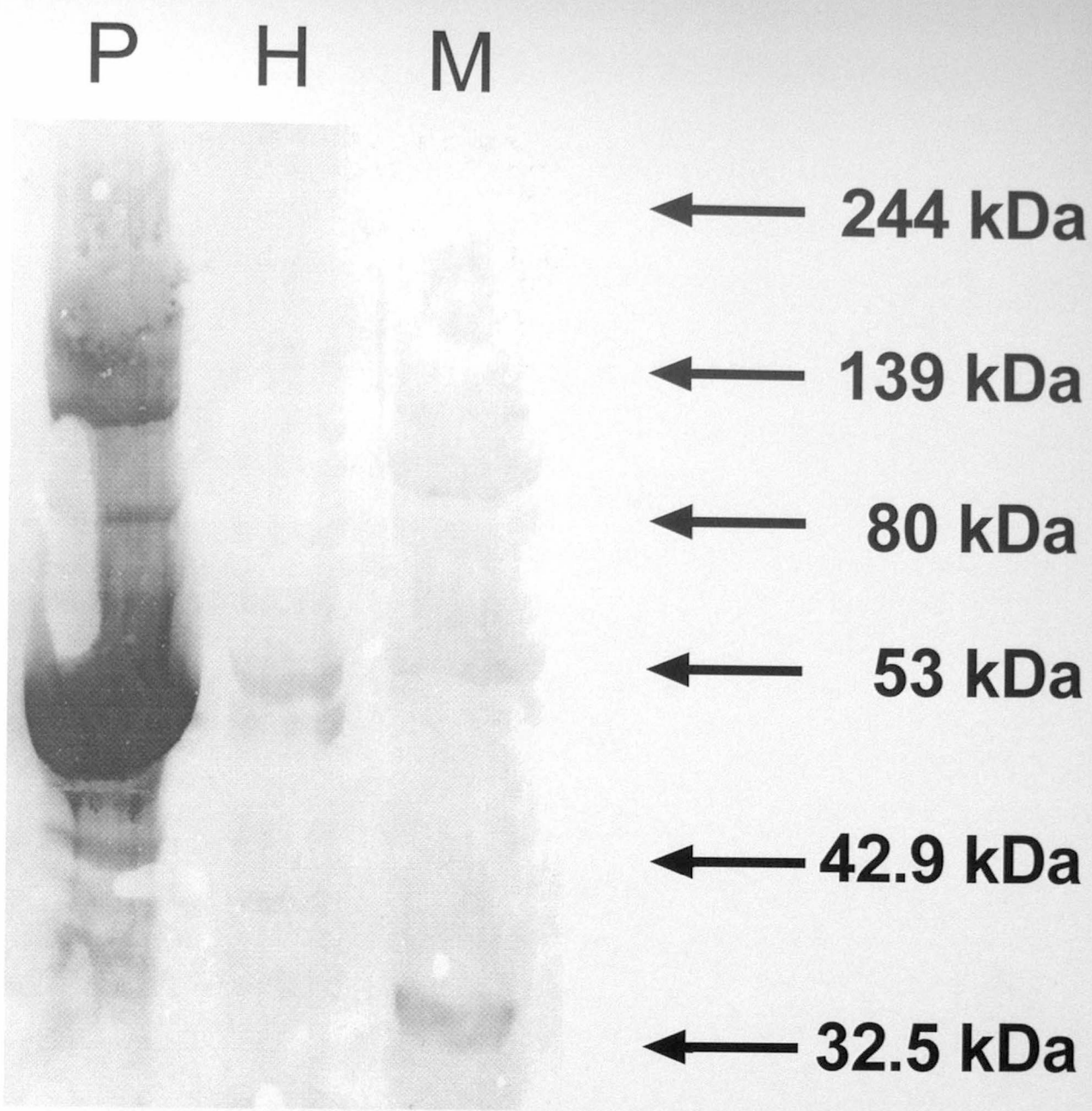


Figure 6.6. Western blot of the cytoplasmic extracts of *S.Pneumoniae* type III (P), Hib (H), and *N.Meningitidis* (M) probed with an anti-pneumolysin antisera.

- Despite the fact that this result was repeated in a second gel, it is possible that the results presented here were due to contamination of tracks H and M with protein from the overloaded P track. Therefore further confirmatory investigations are necessary.

6.7 Meningococcal Extract : Overall Conclusions

- Electrophysiological evidence revealed some ototoxic activity within the meningococcal extract. However, the results were not consistent between animals.

- Protein analysis revealed the presence of a 'pneumolysin-like' protein in the meningococcal extract along with a smaller polypeptide which may be a degradation product of the 'pneumolysin-like' protein.

- **IF** the 'pneumolysin-like' protein revealed by protein analysis is an ototoxin, the protein analysis data indicated that this ototoxin was low in concentration and may have undergone degradation during the disruption process. This would explain the inconsistent ototoxicity produced by the extract in different animals.

- In summary there may be a low concentration of an ototoxin within the meningococcal extract, which may be a 'pneumolysin-like' protein, but further confirmatory investigations are needed.

CHAPTER 7

Characteristics Of Ototoxicity In Crude Meningococcal Bacterial Disruptate.

Although ototoxicity was not consistently demonstrated by the crude meningococcus bacterial disruptate, I have nonetheless attempted below to characterise such ototoxicity as was produced by this extract.

7.1 Electrophysiological Observations

Extracts of the crude meningococcus bacterial disruptate produced ototoxicity in four animals. In three out of four animals the ototoxicity produced was very similar in both potency and timing (see animals 2, 3 and 7). A positive loss in the CAP was only seen at the 10kHz frequency and was between 20 and 27 dB. The loss in the CAP followed a gradual decline and reached a positive level by 30 min. There were no positive losses seen in the CM. In contrast, ototoxicity produced in the fourth animal (animal 9) was much more potent and fast acting. Positive loss was seen in the CAP at all frequencies and in the CM. All losses were achieved within 5 min of perfusion and continued at this level throughout. The CAP was completely eliminated, and the CM loss reached 93%. It is possible that the data from this one animal reflects an experimental error. Since the result produced in this one experiment was not repeated, the data should be treated with caution.

7.2 Morphological Observations

Cochleae from animals 2, 3 and 9 which all exhibited ototoxicity in terms of electrophysiological responses were examined for morphological damage by scanning electron microscopy **(Plates 7 i-ii).**

The treated cochlea from **Animal 2** was curled in many places which meant examination of the organ of Corti was not possible. However, there were some areas which had not curled and these were examined. There was no indication damage to the surface of the organ of Corti at the base of

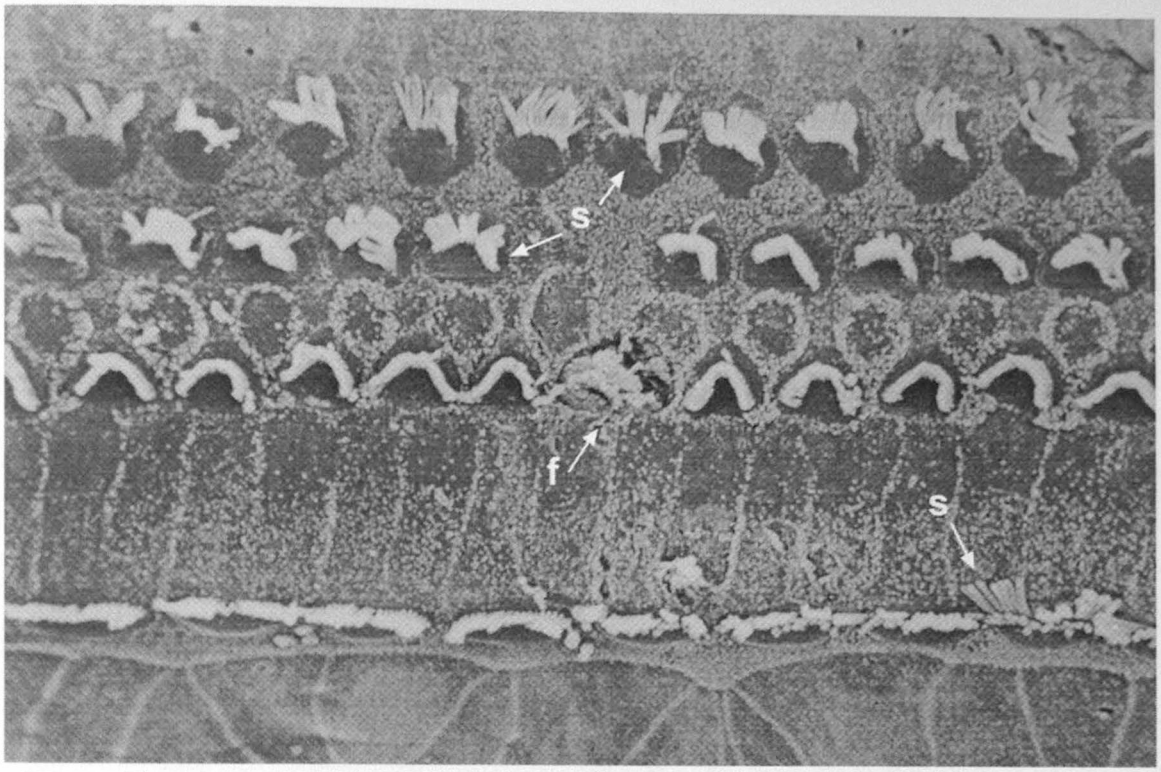


Plate 7i. Low power SEM of the second turn of the organ of Corti from the left cochlea of animal 2 treated with meningococcal extract. There is some splaying (s) of the stereocilia of IHCs and some OHCs of rows 2 and 3. There is also some fusing (f) of the sterocilia of the OHCs in row 1. Magnification x 1500

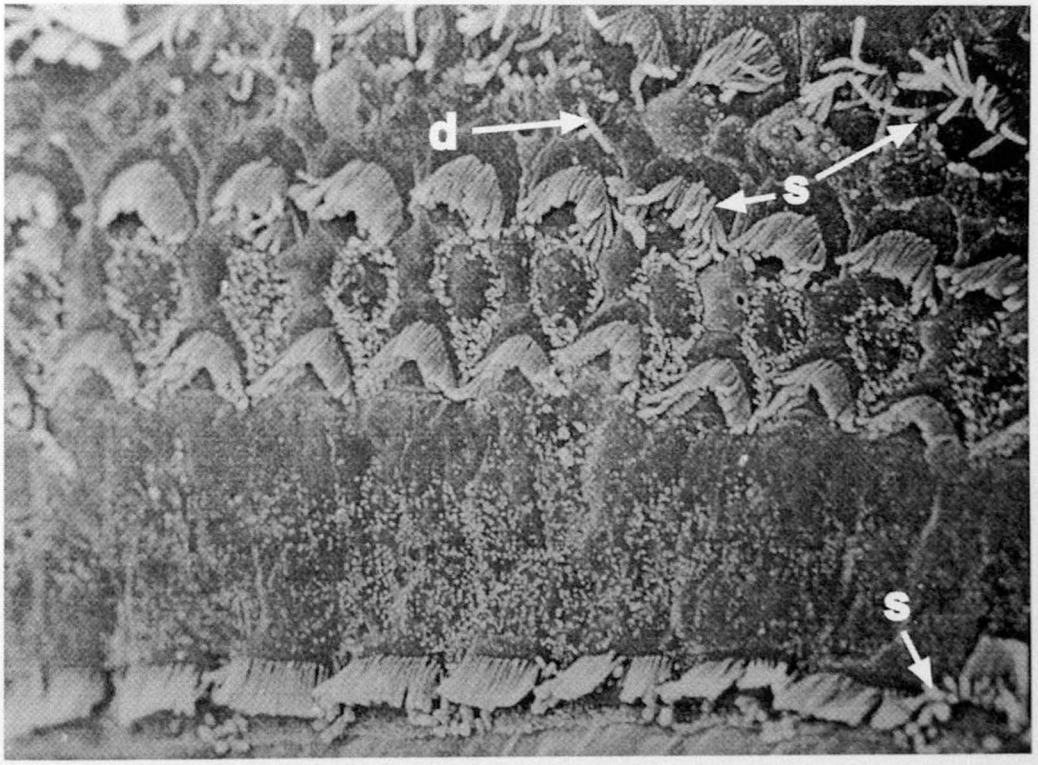


Plate 7ii. High power SEM of the left cochlea from animal 9 treated with meningococcal extract showing some splaying (s) of the stereocilia of IHCs and OHCs of row 1, and severe splaying (s) of the stereocilia of OHCs in rows 2 and 3 with some stereocilia detached from the cell (d). Magnification x 2250

the cochlea, however there was some patchy damage in the second turn (up from the base) of the cochlea. Stereocilia of the inner and outer hair cells were disarrayed and fused in some cases. There were also some craters in the supporting cells.

There was no indication of damage to the surface of the organ of Corti of the treated cochlea from **Animal 3**. However, examination of the organ of Corti was not possible at some sites due to curling of the specimen.

In the cochlea from **Animal 9,** there was evidence of damage to the organ of Corti which involved splaying of the stereocilia of outer and inner hair cells, and cratering of the supporting cells. However, this result was from one patch of the organ of Corti in the second turn because examination of the organ of Corti was not possible at any other sites due to curling of the specimen.

Characteristics Of Ototoxicity In Crude Meningococcal Bacterial Disruptate : Conclusions

- There was some evidence of surface morphological damage which involved splaying of stereocilia of all hair cells, and cratering in supporting cells. Since the damage seen in animal 2 was patchy, the lack of discernible damage in animal 3 may be due to the inability to see all of the organ of Corti rather than the absence of morphological damage.
- Curling of the specimens appeared to be at least partly due to extract treatment as control cochleae which had been prepared identically displayed far less curling, and the degree of curling was consistent with CAP losses.
- The electrophysiological data suggest that the major target of the Meningococcal "ototoxic factor" appears to have been to the inner hair cells, afferent synapse, or afferent neurons and not the outer hair cells, since in most cases the CAP was reduced without any loss to the CM. However, the morphological evidence suggests that the Meningococcal "ototoxic factor" also produced some damage to the outer hair cells and supporting cells.

CHAPTER 8

Ototoxicity Of *Streptococcus pneumoniae* Type III Bacterial Extracts

An initial pilot study in our group suggested that there may be another ototoxic factor within the bacterial extract of *S.pneumoniae* type III besides pneumolysin, the expression of which may be interrupted by the insertional mutation in 'non-pneumolysin producing' pneumococcal mutants (Comis 1992 unpublished data). In the present study similar experiments to those carried out in the pilot study were performed with additional protein assays in an attempt to confirm the existence of this potentially novel ototoxin. Identification and characterisation of ototoxicity was carried out by electrophysiological and morphological studies and appropriate protein assays.

Pneumococcal extracts were provided by Prof. T.J. Mitchell from Leicester (now Glasgow) University. Bacteria were grown and disrupted at Leicester and transported to Birmingham for assessment of ototoxicity. Experiments were carried out on two batches of extracts. Results from batches I and II are presented in sections 8A and 8B respectively. In total 30 animals were used in the work described in this section and the initial auditory thresholds of all these animals can be seen in **Fig. 8**. Three animals were excluded from statistical and summary information, two displayed initial auditory thresholds above the defined level, and a third animal displayed initial auditory thresholds which were considered to be well above the normal distribution of the group.

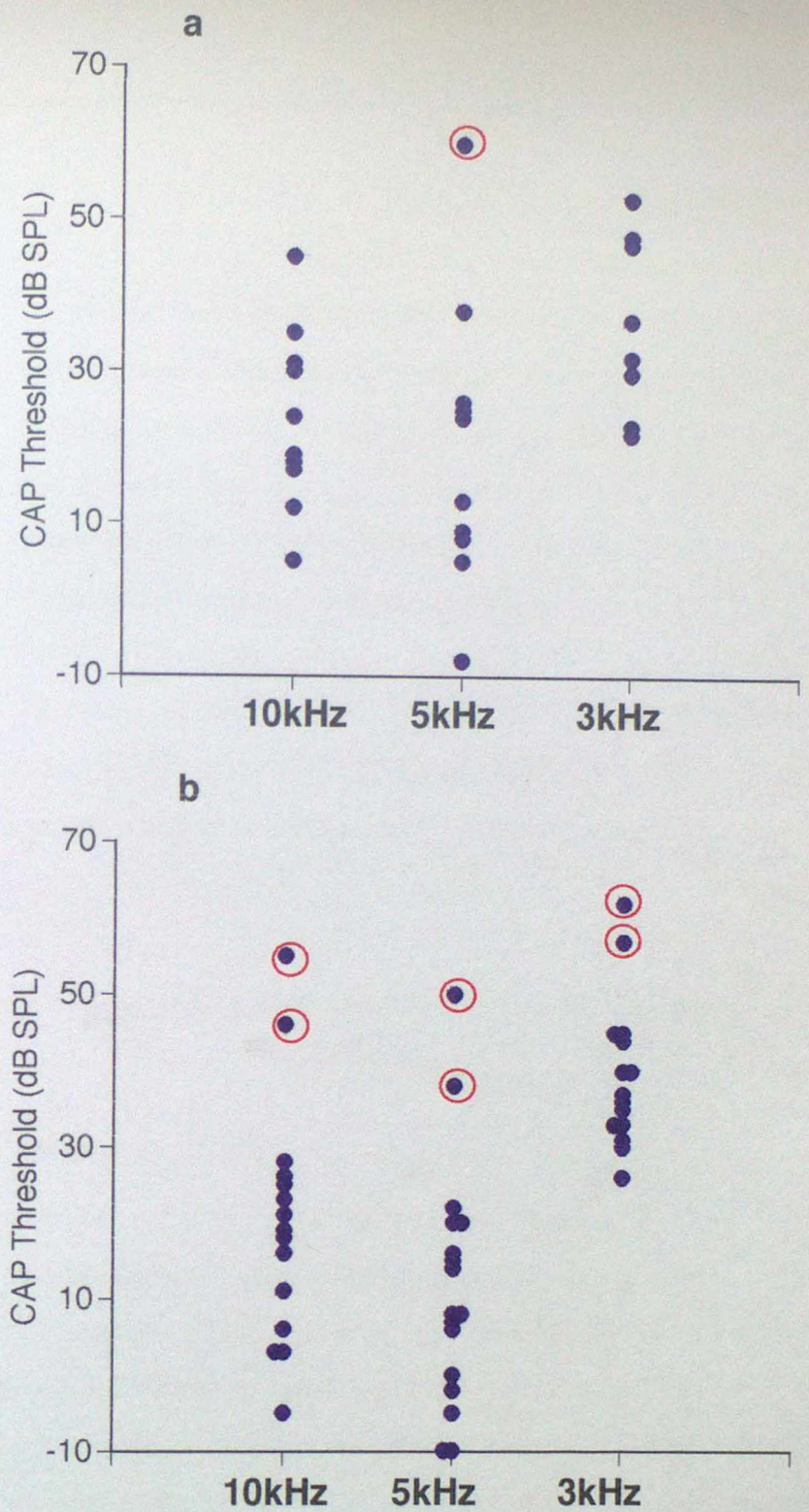


Figure 8. Threshold CAP values of all animals used in the work described in this chapter.

a) Animals perfused with Batch I extracts b) animals perfused with Batch II extracts
Red circles indicate thresholds either above the defined level
(50dB at 10 and 5kHz and 65dB at 3kHz),
or well outside the normal distribution of the group.

Aims and Objectives

1. To perfuse three kinds of pneumococcal extracts derived from *S.pneumoniae* type III into scala tympani of guinea pig cochleae. These extracts were: wild type pneumococcal extract; wild type pneumococcal extract immunopurified to remove pneumolysin; and an extract from *S.pneumoniae* type III in which the pneumolysin gene had been inactivated.

2. To measure electrophysiological responses to test for ototoxicity in each extract.

3. To carry out morphological examinations of those treated cochleae which demonstrated ototoxicity in terms of electrophysiological responses.

8A Ototoxicity Of Pneumococcus Type III Bacterial Extracts: BATCH I

All extracts from batch I are identified by (I). Each batch I extract was initially tested for ototoxicity in one animal. Protein analysis revealed that the protein concentrations within each extract were variable (Wild Type - 1.102 $mgml^{-1}$, Immunopurified Wild Type - 0.3508 $mgml^{-1}$ and Mutant - 0.5263 $mgml^{-1}$). The immunopurified extract (I) contained the least amount of protein, therefore wild type and mutant extracts were diluted to the same concentration (0.3508 $mgml^{-1}$). Data from undiluted wild type and mutant extracts are presented, but are excluded from statistical and summary information as they are not strictly comparable.

8A 1 Wild Type Pneumococcal Extract

8A 1.1 Undiluted Wild Type Pneumococcal Extract (I).

The neat (1.10 $mgml^{-1}$) wild type extract (I) was tested for ototoxicity in animal 1 and produced large losses in CAPs and CMs. All recordable CAPs were eliminated, and the CM was reduced by 80% at 90 min (**Fig. 8A 1**; **Table 8A 1.1**). Data from this animal are excluded from statistical and summary information as the protein concentration was not standardised, and the initial auditory thresholds of this animal were outside the defined range.

Table 8A 1.1 : Electrophysiological losses evoked by perfusion with neat wild type pneumococcal extract (I) in one animal. Positive losses are bold.

Time post perfusion (min)	Animal 1			
	CAP			CM
	10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) Peak CM (mV)			
	5	60	-	0.12
	Losses in CAPs (dB) and CM (% control)			
1	**84**	**20**	-	23
5	**85**	**21**	-	29
10	**90**	**30**	-	**51**
30	**90**	**30**	-	**77**
60	**90**	**30**	-	**77**
90	**90**	**30**	-	**80**

8A 1.2 Diluted Wild Type Pneumococcal Extract (I)

Diluted (0.35 $mgml^{-1}$) wild type pneumococcal extract (I) was tested for ototoxicity in three animals (**Fig. 8A 1**; **Table 8A 1.2**) and produced positive CAP losses in animals 2 and 3 at all frequencies, but not in animal 4. At 90 min, 10kHz CAP losses were 26, 60 and 8 dB in animals 2, 3 and 4 respectively. A positive CM loss of 54% was displayed by animal 3 only. However, 90 min CM loss in animal 10 was 37% which is just below the significance level.

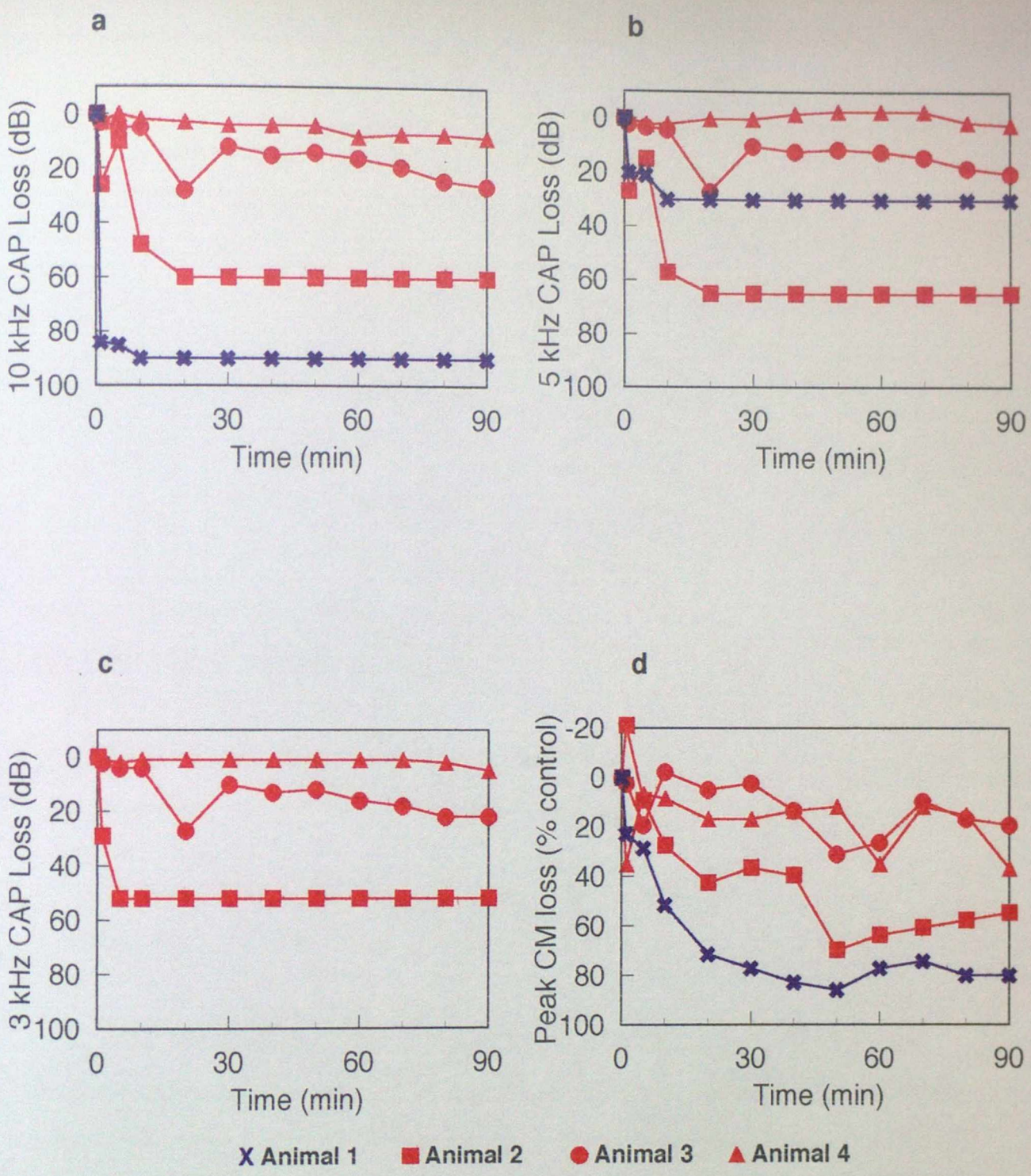


Figure 8A 1. Losses in the CAP and CM in four animals, one treated with neat wild type pneumococcal bacterial extract (BLUE) and three treated with diluted wild type pneumococcal bacterial extract (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 8A 1.2 : Electrophysiological losses evoked by perfusion with diluted wild type pneumococcal extract (I) in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 2				Animal 3				Animal 4			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	30	24	47	0.15	35	25	53	0.12	17	8	30	0.21
	Loss in CAPs (dB) or CM (% control)											
1	3	2	2	2	**26**	**27**	**29**	-21	3	0	1	35
5	5	3	4	19	10	15	**52**	9	0	2	2	7
10	5	4	4	-2	**48**	**57**	**52**	27	2	2	1	8
30	12	10	10	2	**60**	**65**	**52**	36	4	0	1	17
60	16	12	16	26	**60**	**65**	**52**	**64**	8	-3	1	35
90	**26**	**20**	**22**	19	**60**	**65**	**52**	**54**	8	2	5	37

8A 1.3 Wild Type Pneumococcal Extract : Conclusions

- There was evidence for potent ototoxicity (as judged by CAP losses >40dB) in neat wild type pneumococcal extract despite high initial auditory thresholds. However, this result was from just one animal.
- There was some evidence for ototoxicity in diluted wild type pneumococcal extract (I). However the results were not consistent between animals.

8A 2 Immunopurified Pneumococcal Wild Type Extract (I)

Wild type pneumococcal extracts contain the pneumolysin ototoxin. An immunopurified wild type extract from which pneumolysin had been removed was assessed for ototoxicity in order to establish whether or not pneumolysin is responsible for all ototoxicity produced by wild type pneumococcal extracts. Neat (0.35 $mgml^{-1}$) immunopurified wild type extract (I) was tested for ototoxicity in animals 5, 6 and 7 and produced some positive CAP losses at all frequencies, in all animals. Animal 7 displayed mostly transient CAP and CM losses, however, at 90 min, a positive loss was seen in the 5kHz CAP. At 90 min, 10kHz CAP losses were 49, 25 and 16 dB in animals

5, 6 and 7 respectively. In Animal 5, the CM was almost completely eliminated, whereas animal 6 exhibited no positive CM losses. Animal 7 displayed mostly transient CM losses (**Fig. 8A 2**; **Table 8A 2**).

Table 8A 2 : Electrophysiological losses evoked by perfusion with neat immunopurified wild type pneumococcal extract (I) in three animals. Positive losses are bold.

Time post perfusion (min)	Animal 5				Animal 6				Animal 7			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	45	38	53	0.12	19	9	32	0.21	12	-8	23	0.22
	Loss in CAPs (dB) or CM (% control)											
1	10	2	0	-7	-8	-5	0	-11	4	-1	0	-1
5	3	11	6	29	-6	6	6	-7	0	2	2	-3
10	1	3	0	-3	1	10	9	-10	5	3	3	-1
30	4	4	0	**68**	18	**23**	19	3	4	5	2	-3
60	**48**	**48**	**33**	**85**	**23**	**26**	**24**	12	11	9	5	-3
90	**49**	**52**	**39**	**97**	**25**	**27**	**27**	18	16	**21**	12	29

Conclusions

- There was evidence for **sustained** ototoxicity in immunopurified wild type pneumococcal extract (I).
- In addition, there was some evidence for **transient** ototoxicity at 40 min in immunopurified wild type pneumococcal extract (I). However the results were not consistent between animals.

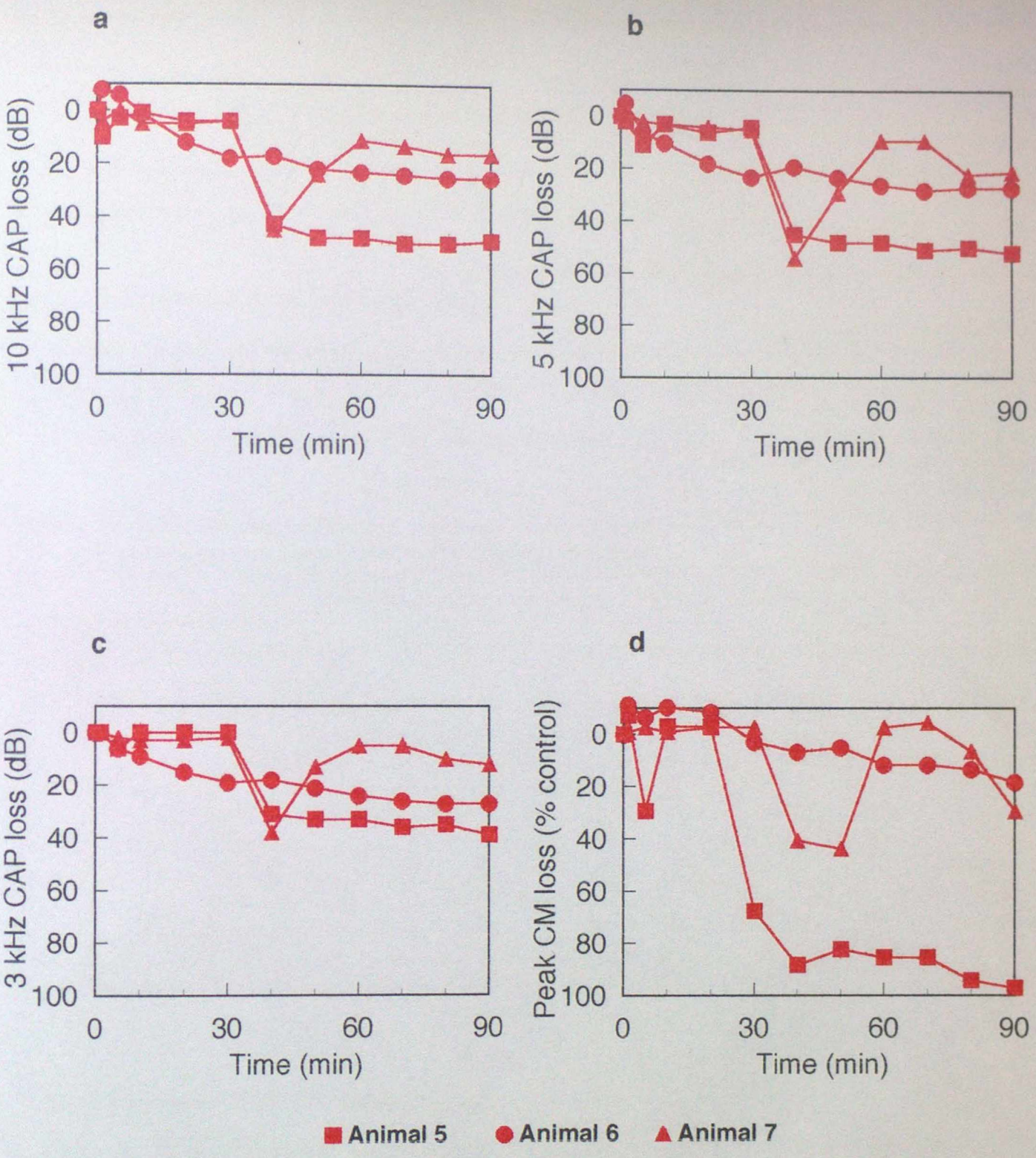


Figure 8A 2. Losses in the CAP and CM in three animals, each treated with neat immunopurified wild type pneumococcal bacterial extract.

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

8A 3 Mutant Pneumococcal Extract (I)

The next step was to determine whether ototoxicity was present in bacterial extract (I) of a mutant of pneumococcus type III which is unable to produce pneumolysin.

8A 3.1 Undiluted Mutant Pneumococcal Extract (I)

Neat (0.53 $mgml^{-1}$) mutant extract (I) was tested for ototoxicity in animal 8 and produced positive CAP and CM losses at 1 min (**Fig. 8A 3**; **Table 8A 3.1**). No further positive CAP or CM losses were displayed, indicating a temperature effect (as discussed in Section 3.1.).

Table 8A 3.1 : Electrophysiological losses evoked by perfusion with neat mutant pneumococcal extract (I) in one animal.
Positive losses are bold.

Time post perfusion (min)	Animal 8			
	CAP			CM
	10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) Peak CM (mV)			
	18	13	37	0.15
	Losses in CAPs (dB) and CM (% control)			
1	**20**	11	8	**56**
5	0	0	12	33
10	1	1	2	2
30	5	6	4	12
60	5	6	3	7
90	6	6	5	12

8A 3.2 Diluted Mutant Pneumococcal extract (I)

Diluted (0.35 $mgml^{-1}$) mutant extract (I) was tested for ototoxicity in animals 9 and 10 and produced positive CAP losses in both animals. However, animal 9 displayed positive CAP losses at 1 min only, indicating a temperature effect (as discussed in Section 3.1.). At 90 min, 10kHz CAP losses were 9 and 32 dB in animals 9 and 10 respectively. A positive CM loss was displayed by animal 9 but not by animal 10 (**Fig. 8A 3**; **Table 8A 3.2**).

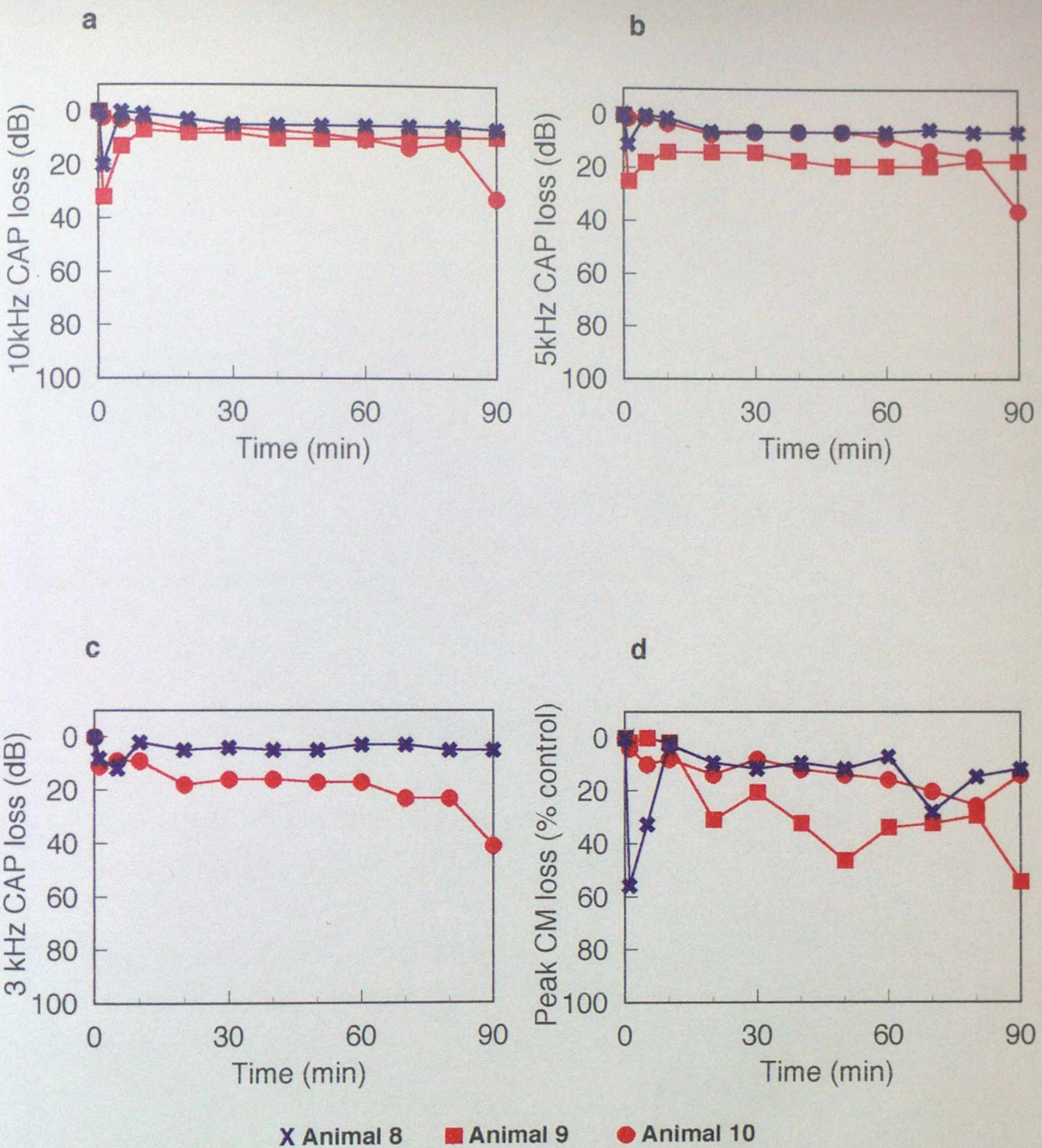


Figure 8A 3. Losses in the CAP and CM in three animals, one treated with neat mutant pneumococcal bacterial extract (BLUE) and two treated with diluted mutant pneumococcal bacterial extract (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 8A 3.2: Electrophysiological losses evoked by perfusion with diluted mutant pneumococcal extract (I) in two animals.
Positive losses are bold.

Time post perfusion (min)	Animal 9				Animal 10			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	31	26	48	0.24	24	5	22	0.18
	Loss in CAPs (dB) or CM (% control)							
1	**32**	**25**	-	2	2	1	11	4
5	13	18	-	0	3	1	9	10
10	7	14	-	2	4	3	9	8
30	8	14	-	21	6	6	16	8
60	10	19	-	34	10	8	17	16
90	9	17	-	**54**	**32**	**36**	**41**	14

8A 3.3 Mutant Pneumococcal Extract (I) : Conclusions

- There was no evidence for authentic ototoxicity in the **undiluted** mutant pneumococcal extract (I). However this result was from **one** animal only.

- There was evidence for ototoxicity in the **diluted** mutant pneumococcal extract (I). However, the type of ototoxicity produced (i.e. CAP or CM loss) by this extract was not consistent between animals.

8A 4 Ototoxicity Of Pneumococcal Bacterial Extracts (I) : Statistics

A summary of the mean (± standard deviation) losses in CAPs and CMs produced by batch I pneumococcal extracts can be seen in **Fig. 8A 4** and **Table 8A 4**.

Table 8A 4 : Average 90 min CAP and CM losses evoked by perfusion with pneumococcal extracts (I) (standardised by protein concentration).
Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 90 min post perfusion			
	10 kHz CAP	5 kHz CAP	3kHz CAP	CM
Wild Type (n=3)	**31 ± 26**	**29 ± 32**	**26 ± 24**	37 ± 18
Immunopurified Wild Type (n=3)	**30 ± 17**	**33 ± 16**	**26 ± 14**	**48 ± 43**
Mutant (n=2)	**20 ± 6**	**26 ± 13**	**41**	34 ± 29

Statistical analysis by ANOVA demonstrated no significant difference between the 10kHz CAP losses produced by each of the pneumococcal extracts.

Immunopurified vs. Mutant	Treatment alone	p= 1.6
	Change in the CAP with time	p= 0.3
Immunopurified vs. Wild Type	Treatment alone	p= 0.7
	Change in the CAP with time	p= 0.2
Wild Type vs. Mutant	Treatment alone	p= 0.5
	Change in the CAP with time	p= 0.2

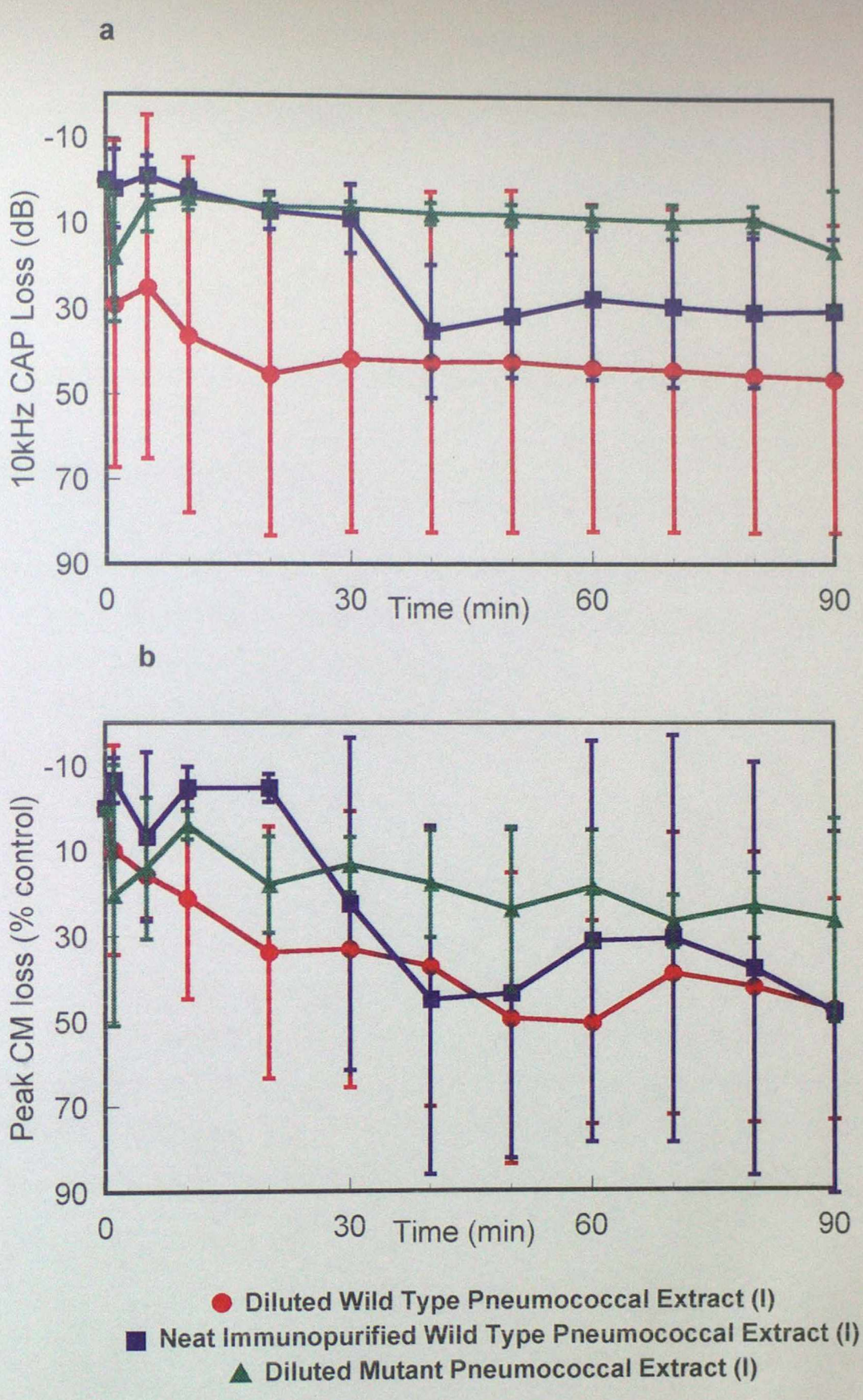


Figure 8A 4. Average losses (± S.D.s) in the CAP and CM produced by three treatment groups.

a Average losses in the CAP elicited with 10 kHzstimuli. b Average losses in the CM elicited with 5kHz stimuli.

8A 5 Ototoxicity Of Pneumococcal Bacterial Extracts (I) : Summary and Conclusions

CAP and CM losses produced by each extract (I) are summarised in **Fig. 8A 5.**

Conclusions

- There was evidence for ototoxicity in immunopurified wild type pneumococcal extract (I). There was some evidence for ototoxicity in wild type, and mutant pneumococcal extracts (I). However the results were not consistent between animals.

- The variability of the animal responses to these preparations could be interpreted to mean that there were no pneumococcal ototoxins. However, it is well established that pneumolysin, a potent ototoxin is present in wild type pneumococcal extracts. Therefore the data could represent a variation in the sensitivity of animals to low concentrations of ototoxin(s). Alternatively, since crude preparations of pneumolysin are thiol-activated, pneumolysin and any other thiol-activated ototoxins present in the pneumococcal extracts (I) may have been inactivated during preparation by oxidation of functional SH groups. Therefore, in part B of this section, further experiments were carried out with a second batch of pneumococcal extracts which were concentrated and DTT-treated to determine further the ototoxicity of these extracts.

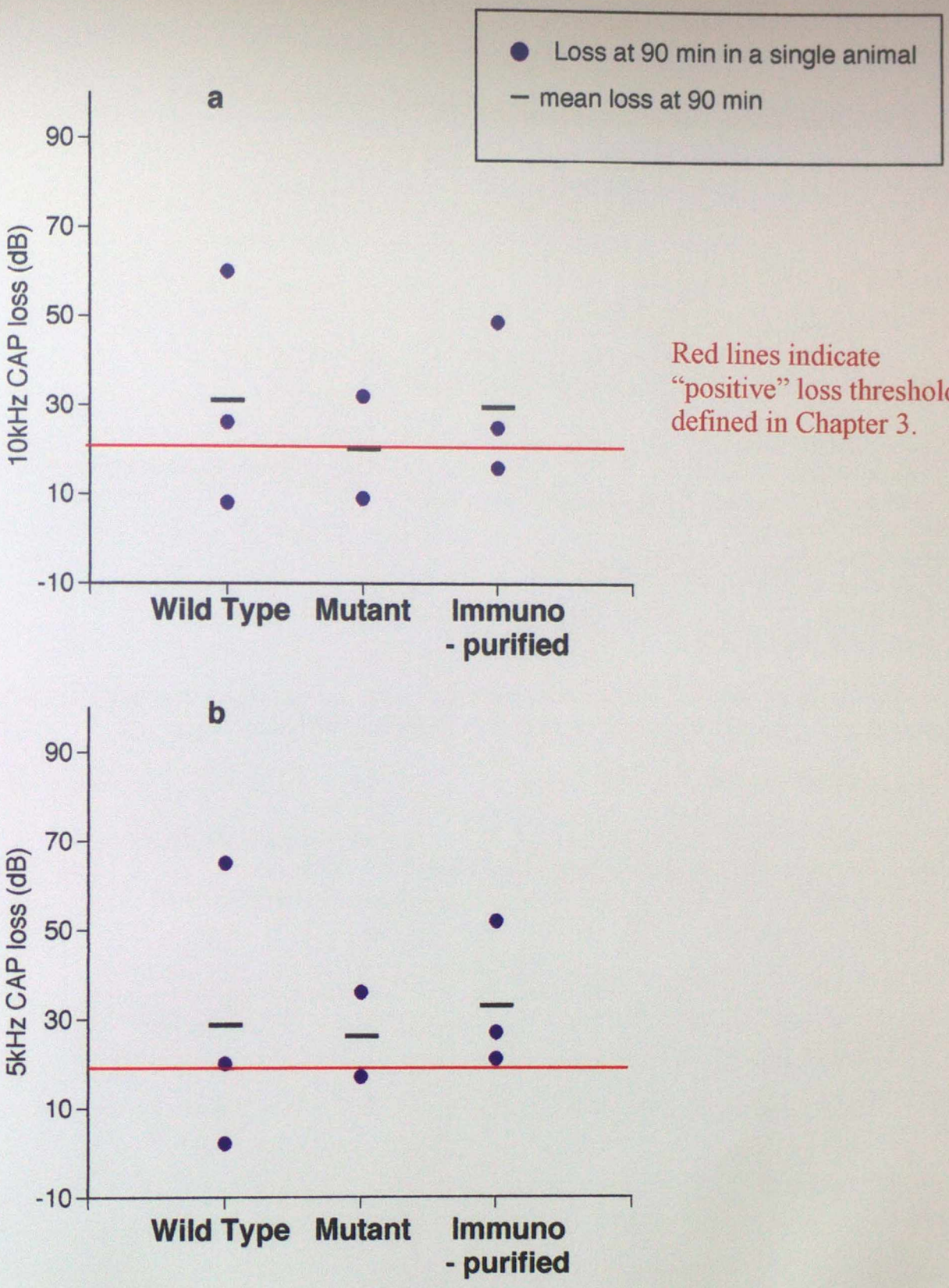


Figure 8A 5. 90 min losses in CAPs and CMs produced by three pneumococcal extracts (I).

a-b) CAP elicited with 10 and 5kHz stimuli respectively.

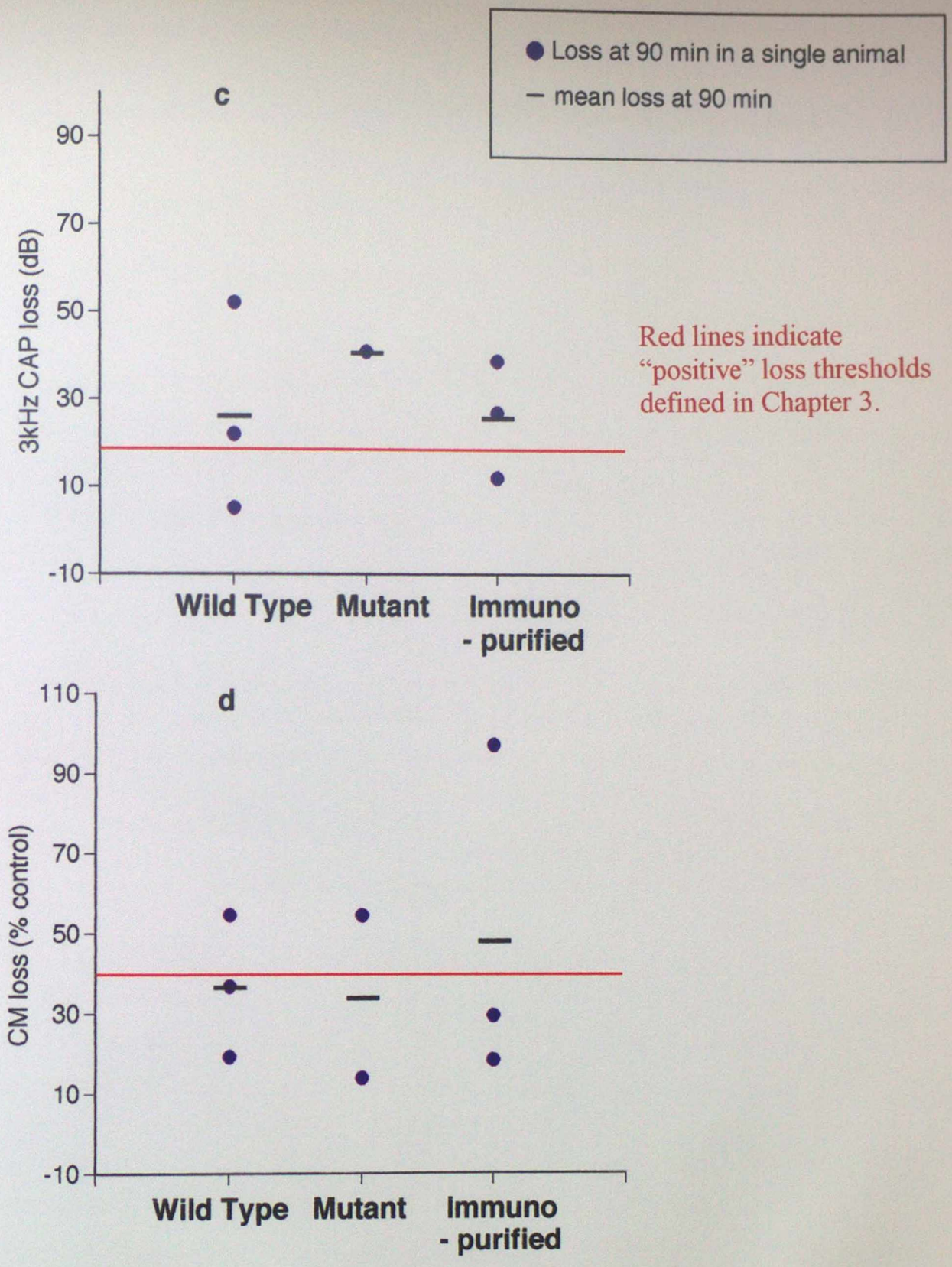


Figure 8A 5. continued. **90 min losses in CAPs and CMs produced by three pneumococcal extracts (I).**

c) CAP elicited with 3kHz stimuli. d) CM elicited with a 5kHz stimuli.

8B Ototoxicity of Pneumococcus Type III Bacterial Extracts: BATCH II

Initially, the neat wild type pneumococcal extract (II) was perfused into the cochlea of one animal, to test for ototoxicity and to compare its activity with the neat wild type extract from batch I.

8B 1 Wild Type Pneumococcal Extract (II)

Wild type pneumococcal extract (II) was tested for ototoxicity in animal 1 (**Fig. 8B 1**; **Table 8B 1**) and produced a positive loss in the 10kHz CAP of 35dB at 10 min. There were no further positive losses in CAPs or CM.

Table 8B 1 : Electrophysiological losses evoked by perfusion with neat wild type pneumococcal extract (II) in one animal.
Positive losses are bold.

Time post perfusion (min)	Animal 1			
	CAP			CM
	10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)			
	18	15	40	0.15
	Loss in CAPs (dB) or CM (% control)			
1	9	3	2	8
5	9	7	5	16
10	**35**	17	12	5
30	11	5	7	5
60	16	5	7	5
90	16	5	7	5

Conclusion

- There was some evidence for **transient** ototoxicity in the wild type pneumococcal bacterial extract (II) .
- There was no evidence for **sustained** ototoxicity in wild type pneumococcal bacterial extract (II) at this concentration.

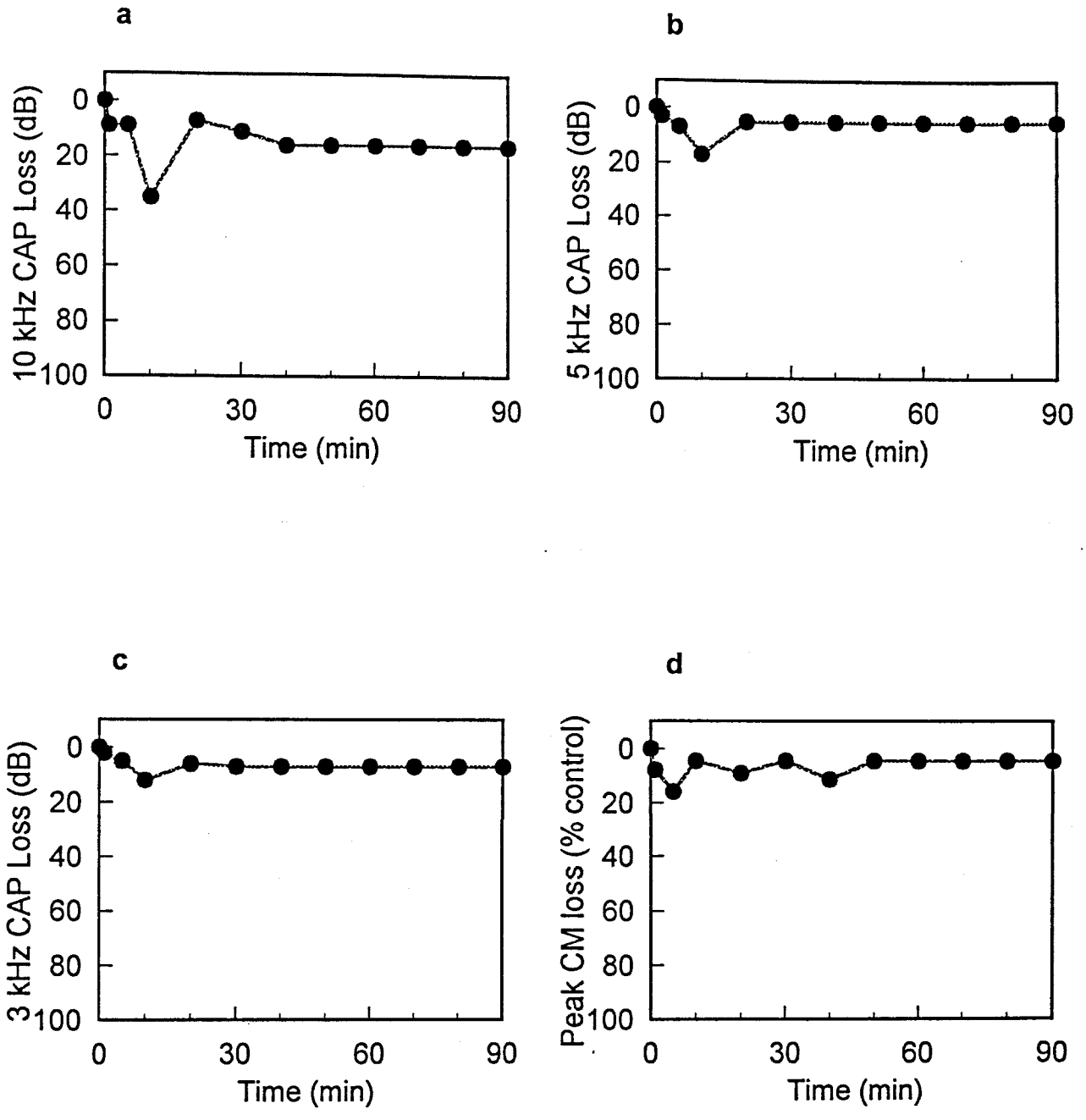


Figure 8 B 1. Losses in the CAP and CM in one animal, treated with neat wild type pneumococcal bacterial extract (II).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

The transient ototoxicity displayed by this animal is reminiscent of the CAP losses produced by perfusion of diluted (0.5µg) pneumolysin (Comis *et al.* 1993).

8B 2 DTT Treatment Of Wild Type Pneumococcal Bacterial extract (II)

In the following experiments wild type pneumococcal bacterial extract (II) was treated with DTT and reassessed for ototoxicity. DTT was added to all extracts to a final concentration of 1mM.

8B 2.1 DTT-Treated BSA

The possible non-specific effects of active thiol groups on the cochleae (see **Section 6.3.1.**), were re-checked as the concentrations needed for comparison were different in this chapter. BSA was used at 2 $mgml^{-1}$ which is the concentration to which all pneumococcal extracts were later concentrated (see section 8B 3). DTT-treated BSA was tested for ototoxicity in animals, 5, 6, 7 and 8 (**Fig. 8B 2.1**; **Table 8B 2.1**). Data from animal 7 are included in Fig. 8B 2.1 and Table 8B 2.1, but are excluded from statistical and summary information as the animal displayed initial auditory thresholds outside the defined range (see Fig. 8). Positive CAP losses were displayed by animals 6 and 8, but not by animals 5 and 7. At 90 min, animal 6 lost 20dB at 10kHz and animal 8 lost 20dB at 5kHz. Positive CM loss of 54% was displayed by animal 5, but not by animals 6, 7 and 8. However, animal 8 displayed a 90 min CM loss of 36% which is just below the significance level.

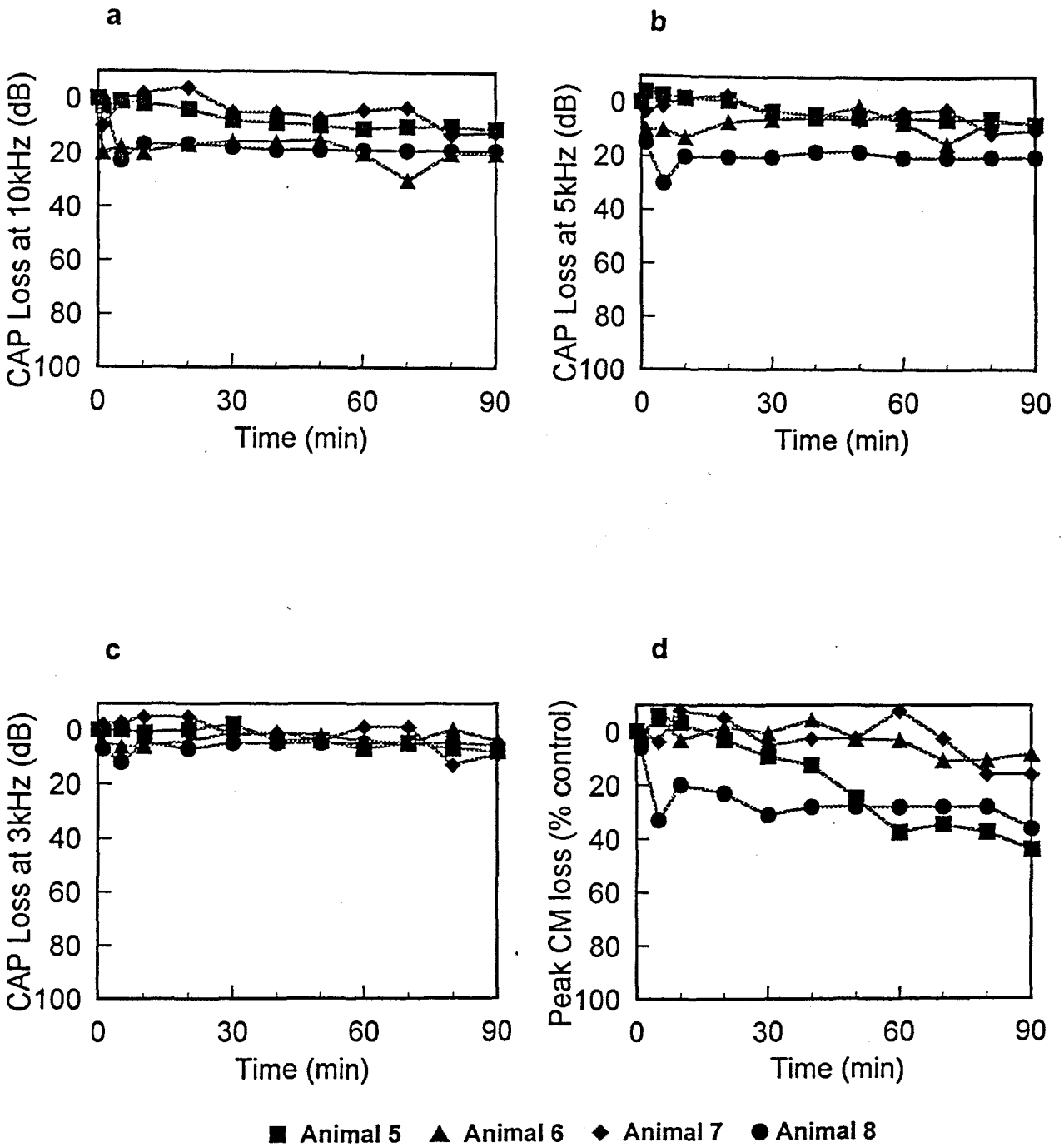


Figure 8B 2.1. Losses in the CAP and CM in four animals, each treated with DTT-treated BSA.

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 8B 2.1 : Electrophysiological losses evoked by perfusion with DTT-treated BSA in four animals. Positive losses are bold.

Time post perfusion (min)	Animal 5				Animal 6			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	19	14	35	0.11	3	-2	33	0.14
	Loss in CAPs (dB) or CM (% control)							
1	3	-4	0	3	**20**	10	5	1
5	1	-3	0	-6	18	10	6	-5
10	2	-2	1	-3	**20**	13	6	3
30	8	3	-2	9	16	6	1	1
60	11	5	7	37	**20**	7	4	3
90	11	8	8	**44**	**20**	7	4	8

Time post perfusion (min)	Animal 7				Animal 8			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	46	38	57	0.13	3	-10	33	0.20
	Loss in CAPs (dB) or CM (% control)							
1	10	3	-2	0	1	15	7	6
5	1	1	-3	4	**23**	**30**	12	33
10	-2	-2	-5	-8	17	**20**	5	20
30	5	5	2	5	18	**20**	5	31
60	4	3	-1	-8	19	**20**	5	28
90	12	10	9	16	19	**20**	6	36

8B 2.2 DTT-Treated Wild Type Pneumococcal Extract

DTT-treated wild type pneumococcal extract was tested for ototoxicity in animals 9 and 10 (**Fig. 8B 2.2; Table 8B 2.2**) and produced positive losses in CAPs at all frequencies in both animals. At 90 min, 10kHz CAP losses were 53 and 36dB (higher than for DTT-treated BSA) in animals 9 and 10 respectively. Losses in CMs were 32 and 33% in animals 9 and 10 respectively which are just below the significance level.

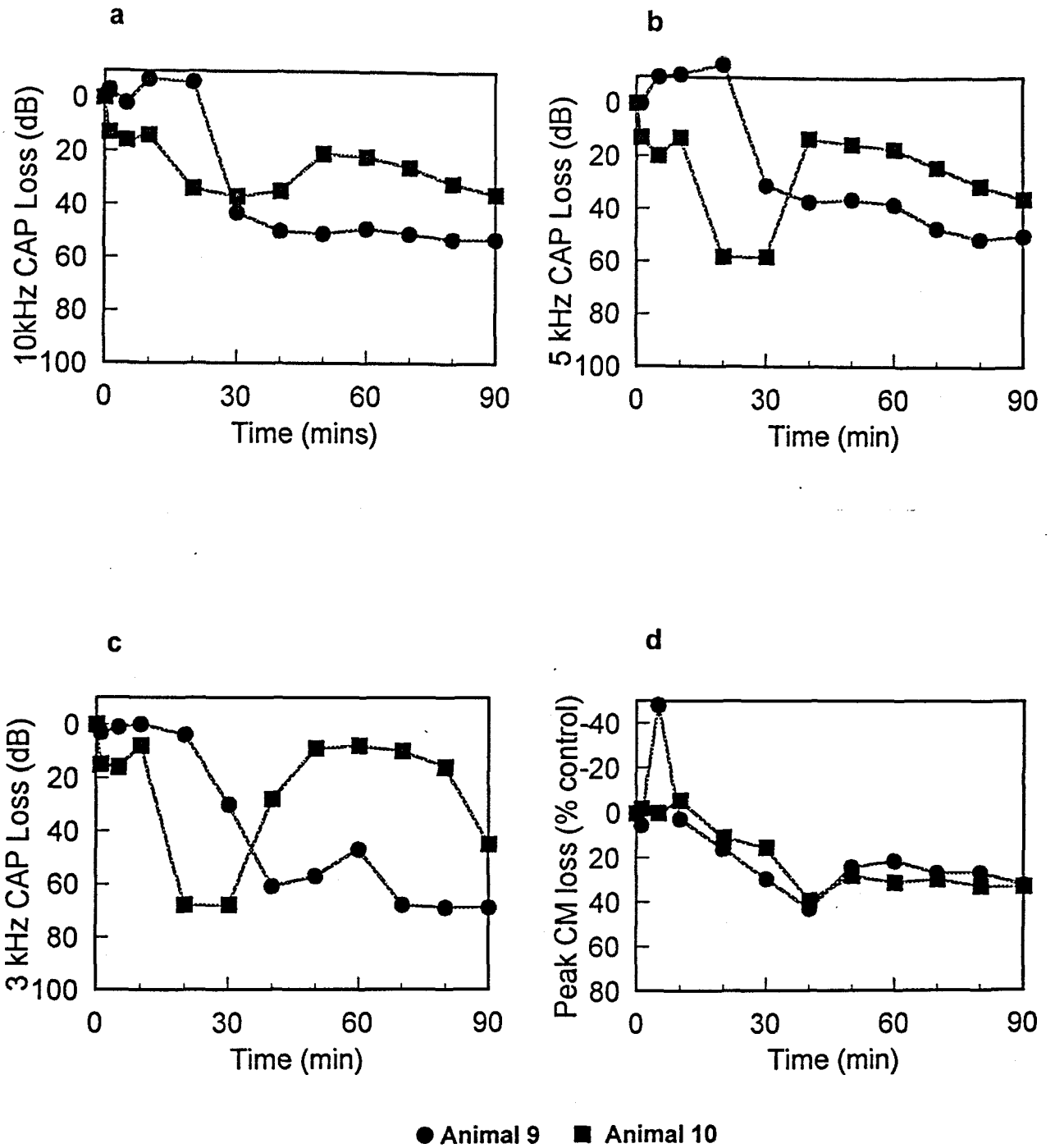


Figure 8B 2.2. Losses in the CAP and CM in two animals, each treated with DTT-treated neat wild type pneumococcal bacterial extract (II)

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 8B 2.2 : Electrophysiological losses evoked by perfusion with DTT-treated neat wild type pneumococcal extract (II) in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 9				Animal 10			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	21	20	36	0.13	16	8	37	0.22
	Loss in CAPs (dB) or CM (% control)							
1	-3	0	3	5	13	13	15	-2
5	2	-10	1	-48	16	20	16	0
10	-7	-11	0	3	14	13	8	-5
30	**43**	**31**	**30**	30	**37**	**58**	**68**	16
60	**49**	**38**	**47**	22	**22**	17	8	32
90	**53**	**50**	**69**	32	**36**	**36**	**45**	33

8B 2.3 DTT-Treated Wild Type Pneumococcal Extract : Summary and Conclusions

Average 90 min losses in CAPs and CMs produced by wild type pneumococcal bacterial DTT-treated extracts (II) , and DTT-treated BSA are summarised in **Table 8B 2.3**.

Table 8B 2.3: Average 90 min losses evoked by perfusion with DTT-treated BSA, neat wild type pneumococcal extract (II) (Neat WT PC extract) and DTT-treated neat wild type pneumococcal extract (II). Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 90 min post perfusion			
	10 kHz CAP	5 kHz CAP	3kHz CAP	CM
Neat WT PC extract (n=1)	16	5	-	5
DTT-treated Neat WT PC extract (n=3)	**44 ± 12**	**43 ± 10**	**57 ± 17**	33 ± 1
DTT-treated BSA (n=4)	17 ± 5	12 ± 7	6 ± 2	30 ± 19

Conclusions

- There was evidence for potent ototoxicity (as judged by CAP losses >40dB) in DTT-treated wild type pneumococcal extract.
- DTT-treated BSA produced some variable low level ototoxicity (as judged by CAP loss) in the guinea pig cochlea. Therefore one may assume that ototoxicity produced by DTT-treated extracts (II) which is consistent between animals is not due to non-specific effects of DTT treatment. However the data presented here demonstrates the need for caution when interpreting the data of extracts treated with DTT.
- Statistical analysis by ANOVA demonstrated a significant difference between the 10kHz CAP losses produced by DTT-treated wild type pneumococcal extract and DTT-treated BSA. The effect of treatment on the CAP at all time points was just outside the significance range ($p=0.05$) but the change in the CAP with time was significant ($p=0.02$).
- DTT treatment considerably enhanced the ototoxicity of the wild type extract (II) (as judged by CAP loss), therefore DTT treatment was routinely carried out in further experiments.

8B 3: Concentrated DTT-Treated Pneumococcal Extracts (II)

Since data from part A of this chapter implied that the ototoxin(s) present in the pneumococcal bacterial extracts may have been low in concentration, all pneumococcal extracts were concentrated before further assessment of ototoxicity. All pneumococcal extracts (II) were concentrated and adjusted to a final protein concentration of $2mgml^{-1}$ and assessed for ototoxicity.

8B 3.1 Concentrated Wild Type Pneumococcal Extract

The concentrated DTT-treated wild type pneumococcal bacterial extract (II) was tested for ototoxicity in animals 11, 12, 13 and 14 (**Fig. 8B 3.1**; **Table 8B 3.1**). The data from animal 14 has been excluded from statistical calculations and summary charts as the animal displayed initial auditory thresholds outside the defined range and was killed at 35 min. In animals 11, 12 and 13

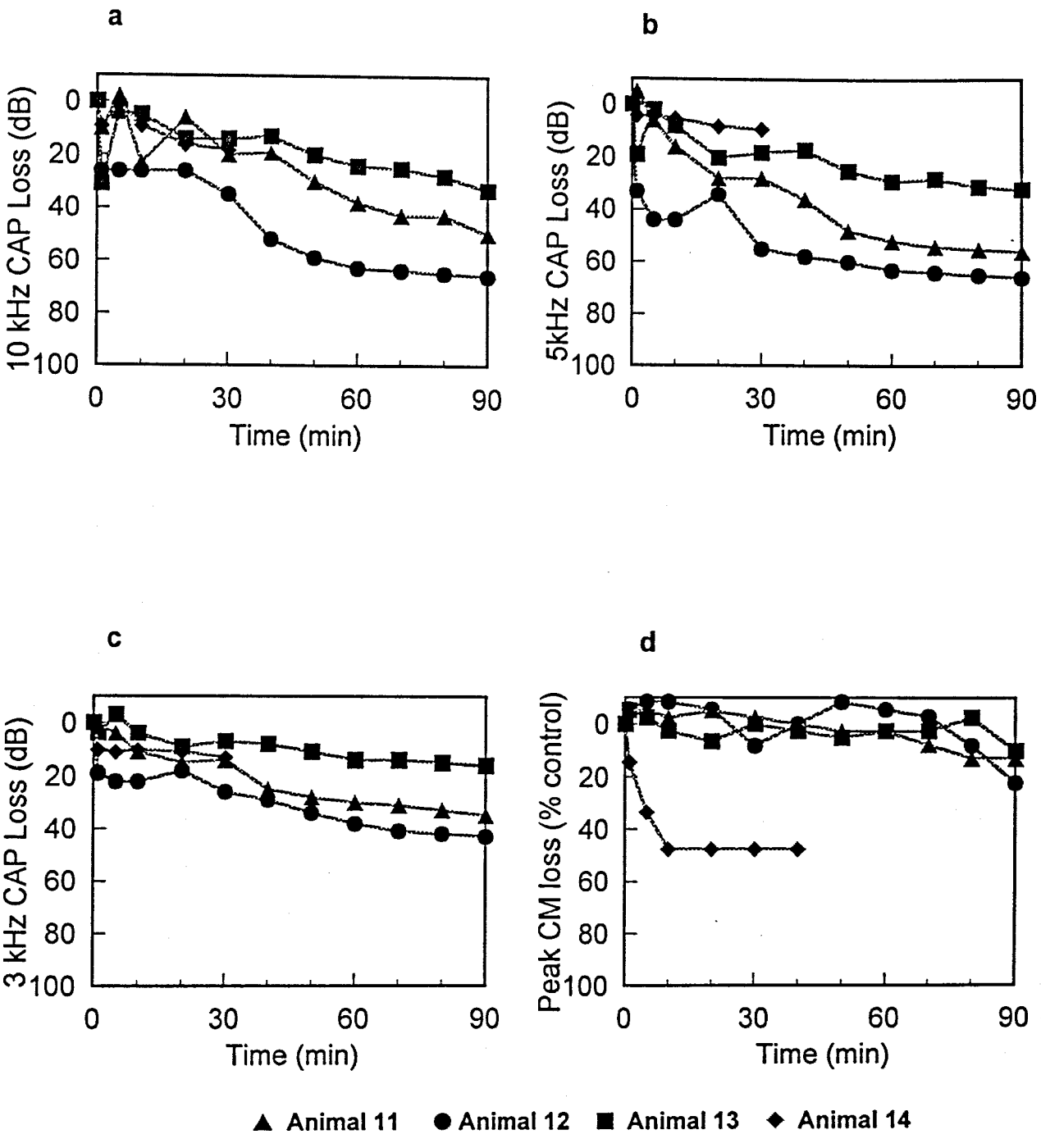


Figure 8B 3.1. Losses in the CAP and CM in four animals, each treated with concentrated DTT-treated wild type pneumococcal bacterial extract (II).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

perfusion of concentrated wild type pneumococcal bacterial disruptate produced positive losses in CAPs at two or more frequencies. Animal 13 displayed a positive 10kHz CAP loss at 1 min which had recovered by 5 min, indicating a temperature effect (see Chapter 3). At 90 min, 10kHz CAP losses were 50, 63 and 33dB in animals 11, 12 and 13 respectively. Animal 14 displayed a 10kHz CAP loss of 18dB at 30 min. A positive CM loss was displayed in Animal 14 of 48%, but not in animals 11, 12 and 13.

Table 8B 3.1 : Electrophysiological losses evoked by perfusion with concentrated and DTT-treated wild type pneumococcal extract (II) in four animals. Positive losses are bold.

Time post perfusion (min)	Animal 11				Animal 12			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	11	-5	30	0.14	6	-10	33	0.13
	Loss in CAPs (dB) or CM (% control)							
1	10	-5	1	-5	**26**	**33**	19	-6
5	-2	6	4	-5	**26**	**44**	**22**	-8
10	**23**	16	11	-2	**26**	**44**	**22**	-8
30	**20**	**28**	14	-2	**35**	**55**	**26**	8
60	**38**	**52**	**30**	3	**63**	**63**	**38**	-6
90	**50**	**56**	**35**	13	**63**	**66**	**43**	22

Time post perfusion (min)	Animal 13				Animal 14			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	26	16	45	0.14	55	50	62	0.07
	Loss in the CAP dB) or CM (% control)							
1	**31**	19	4	-5	9	4	10	14
5	4	2	-3	-2	1	4	11	33
10	5	8	4	3	9	5	10	**48**
30	14	18	7	0	18	9	13	**48**
60	**24**	**29**	14	3	-	-	-	-
90	**33**	**32**	16	10	-	-	-	-

Conclusions

- There was evidence for **sustained** ototoxicity in concentrated wild type pneumococcal extract.
- In addition, there was some evidence for **transient** ototoxicity at 1min in concentrated wild type pneumococcal extract. However, this result was not consistent between animals and is likely to have been due to incomplete equilibration of the sample with ambient temperature.

8B 3.2 Concentrated Immunopurified DTT-Treated Wild Type Pneumococcal Extract.

Perfusion of concentrated and DTT-treated immunopurified wild type pneumococcal extract (II) produced positive CAP losses at two or more frequencies in animals 15, 16 and 18, but not in animal 17 (**Fig. 8B 3.2**; **Table 8B 3.2**). At 90 min, 10kHz CAP losses were 31, 33, 8 and 43 dB in animals 15, 16, 17 and 18 respectively. Animals 15 and 16 displayed some transient CAP losses at 1 min, but both animals displayed further losses, suggesting that the losses may not have been due to a temperature effect There were no positive losses seen in the CM in any animal.

Table 8B 3.2 : Electrophysiological losses evoked by perfusion with concentrated and DTT-treated immunopurified wild type pneumococcal extract (II) in four animals. Positive losses are bold.

Time post perfusion (min)	Animal 15				Animal 16			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	16	7	44	0.17	-5	0	36	0.19
	Loss in CAPs (dB) or CM (% control)							
1	**35**	**27**	6	4	0	8	2	-2
5	17	11	1	4	10	**23**	6	4
10	18	12	1	4	10	18	9	4
30	11	4	-2	8	0	10	4	4
60	**21**	18	0	14	17	7	1	5
90	**31**	**30**	6	16	**33**	**21**	7	9

Table continued overleaf.

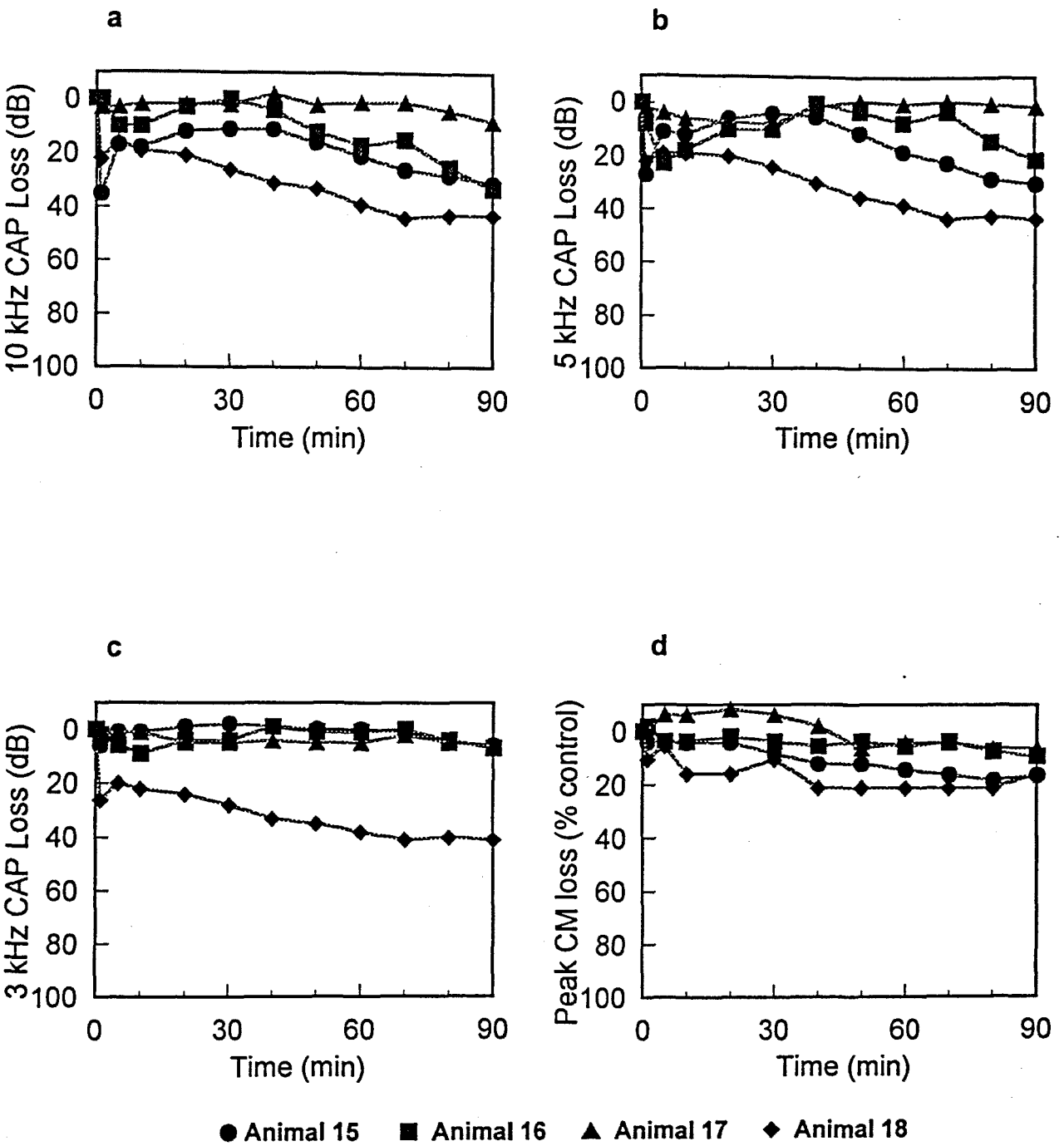


Figure 8 B 3.2. Losses in the CAP and CM in four animals, each treated with concentrated DTT-treated immunopurified pneumococcal bacterial extract (II).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Time post perfusion (min)	Animal 17				Animal 18			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	23	6	26	0.17	28	8	31	0.07
	Loss in CAPs (dB) or CM (% control)							
1	3	2	2	0	**22**	**22**	**26**	11
5	3	4	1	-6	16	19	**20**	5
10	2	6	1	-6	19	19	**22**	16
30	2	8	5	-6	**26**	**24**	**28**	11
60	1	0	5	4	**39**	**38**	**38**	21
90	8	1	6	6	**43**	**43**	**41**	16

Conclusions

- There was evidence for **transient** ototoxicity at 1 min, and later **sustained** ototoxicity in concentrated immunopurified wild type pneumococcal extract. However, neither of these results were consistent between animals.

8B 3.3 : Concentrated 'Non-pneumolysin producing' Mutant Pneumococcal Extract

The concentrated and DTT-treated mutant extract (II) was tested for ototoxicity in animals 19 and 20 (**Fig. 8B 3.3**; **Table 8B 3.3**) and produced positive CAP losses in both animals. Animal 19 displayed a transient CAP loss at 1 min, indicating a temperature effect (as discussed in Section 3.1.). However, at 90 min, losses in the 10kHz CAP were 21 and 38dB in animals 19 and 20 respectively. A positive loss in the CM was not seen in either animal.

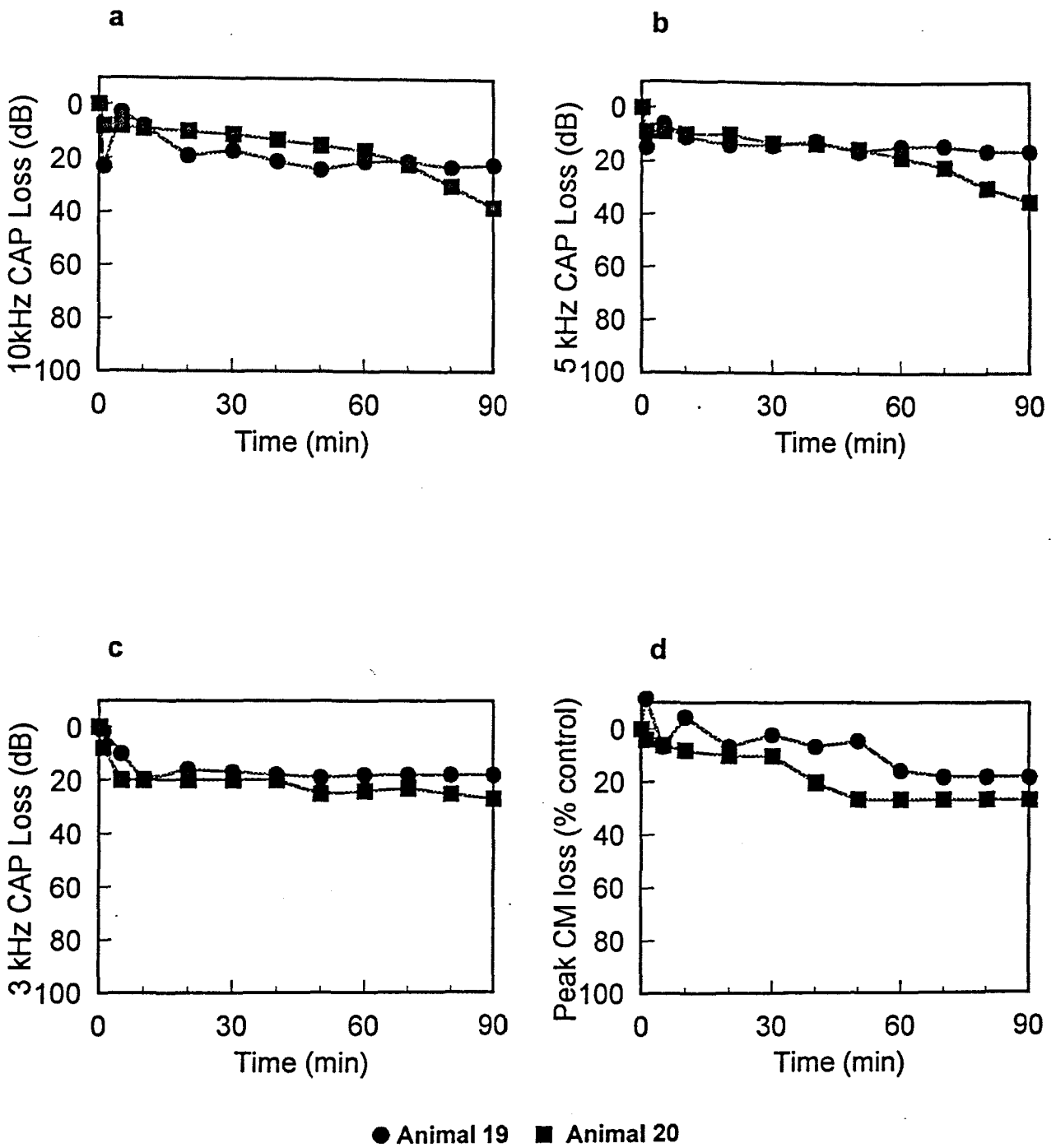


Figure 8B 3.3. Losses in the CAP and CM in two animals, each treated with concentrated DTT-treated mutant bacterial extract (II).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 8B 3.3 : Electrophysiological losses evoked by perfusion with concentrated and DTT-treated mutant pneumococcal extract in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 19				Animal 20			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	26	22	40	0.15	25	20	45	0.21
	Loss in CAPs (dB) or CM (% control)							
1	**23**	15	2	-11	8	9	8	4
5	3	6	10	7	8	9	**20**	6
10	8	11	**20**	-5	9	10	**20**	8
30	17	14	17	2	11	13	**20**	10
60	**21**	14	18	16	17	18	**24**	27
90	**22**	16	18	18	**38**	**35**	**27**	27

8B 3.4 Concentrated DTT-Treated Pneumococcal Extracts (II) : Statistics

Mean (± standard deviations) losses in CAPs and CMs produced by batch II pneumococcal extracts are summarised in **Fig. 8B 3.4** and **Table 8B 3.4.**

Table 8B 3.4: Average losses at 90 min produced by perfusion with DTT-treated BSA, and three concentrated and DTT-treated pneumococcal extracts. Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 90 min post perfusion			
	10 kHz CAP	5 kHz CAP	3kHz CAP	CM
Wild type extract (II) (n=3)	**50 ± 16**	**51 ± 17**	**31 ± 14**	15 ± 6
Immunopurified wild type extract (II) (n=4)	**29 ± 15**	**24 ± 18**	15 ± 17	12 ± 5
Mutant extract (n=2)	**30 ± 11**	**25 ± 13**	**22 ± 6**	22 ± 6
DTT-treated BSA (n=3)	17 ± 5	12 ± 7	6 ± 2	30 ± 19

Statistical analysis by ANOVA demonstrated no statistical significance between all treatment groups with respect to treatment alone. Some statistical significance was demonstrated between

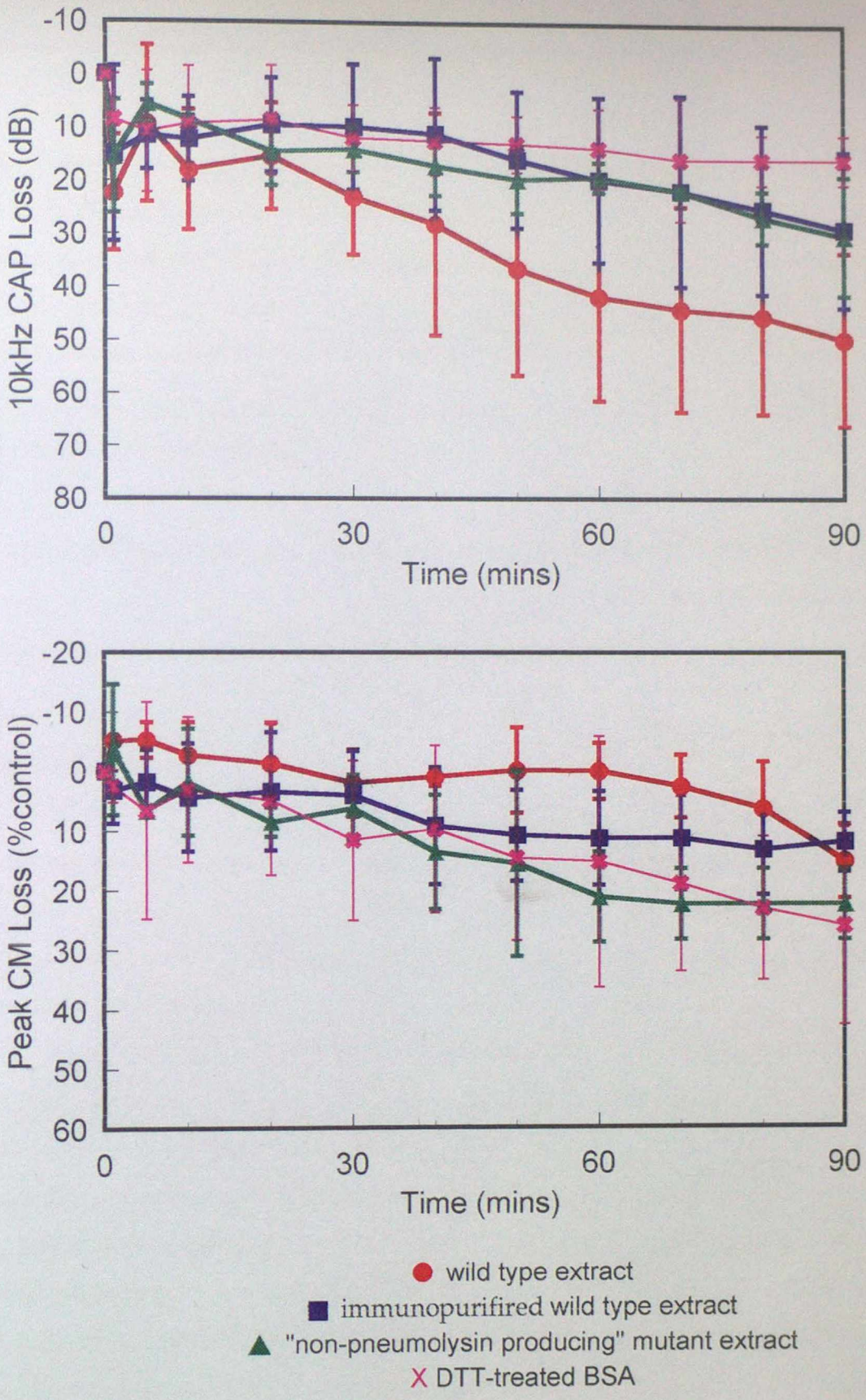


Figure 8B 3.4. Average losses (± S.D.s) in theCAP and CM produced by three concentrated DTT-treated pneumococcal extracts and DTT-treated BSA.

a) Average losses in the CAP elicited with 10 kHzstimuli.
b) Average losses in the CM elicited with 5kHz stimuli.

the data of the wild type pneumococcal extract (II) and the immunopurified wild type extract with respect to the change in the CAP with time ($p=0.03$). A highly significant statistical difference was demonstrated between the wild type extract (II) and DTT-treated BSA with respect to the change in the CAP with time ($p=0.0003$). Therefore immunopurified wild type extract (II) produced significantly less ototoxicity (in terms of 10kHz CAP loss) than unpurified wild type extract. But, only the wild type pneumococcal extract (II) produced ototoxicity that is statistically different to that produced by DTT-treated BSA.

8B 3.5 Ototoxicity Of Pneumococcus Type III Extracts (II) : Conclusions

The scattergram (**Fig. 8B 3.5**) shows more clearly the whole set of data from which one can conclude the following:

- There was evidence for ototoxicity in wild type and mutant pneumococcal bacterial extracts (II) which was consistent between animals. There was some evidence for ototoxicity in immunopurified wild type bacterial extract. However this was not produced in one out of four animals tested.
- Wild type bacterial extract (II) produced more potent ototoxicity (as judged by CAP loss) than both the mutant and immunopurified wild type pneumococcal extracts (II), the latter two preparations produced similar levels of ototoxicity (as judged by CAP loss).
- Therefore there appears to be another ototoxic factor within the bacterial extracts (II) of wild type and mutant pneumococcus type III besides pneumolysin, which may represent a novel ototoxin common to both bacteria.
- However, data from protein analyses, presented in section 8B 4 revealed that the immunopurified extract (II) was contaminated with proteins leached from the column during immunopurification.

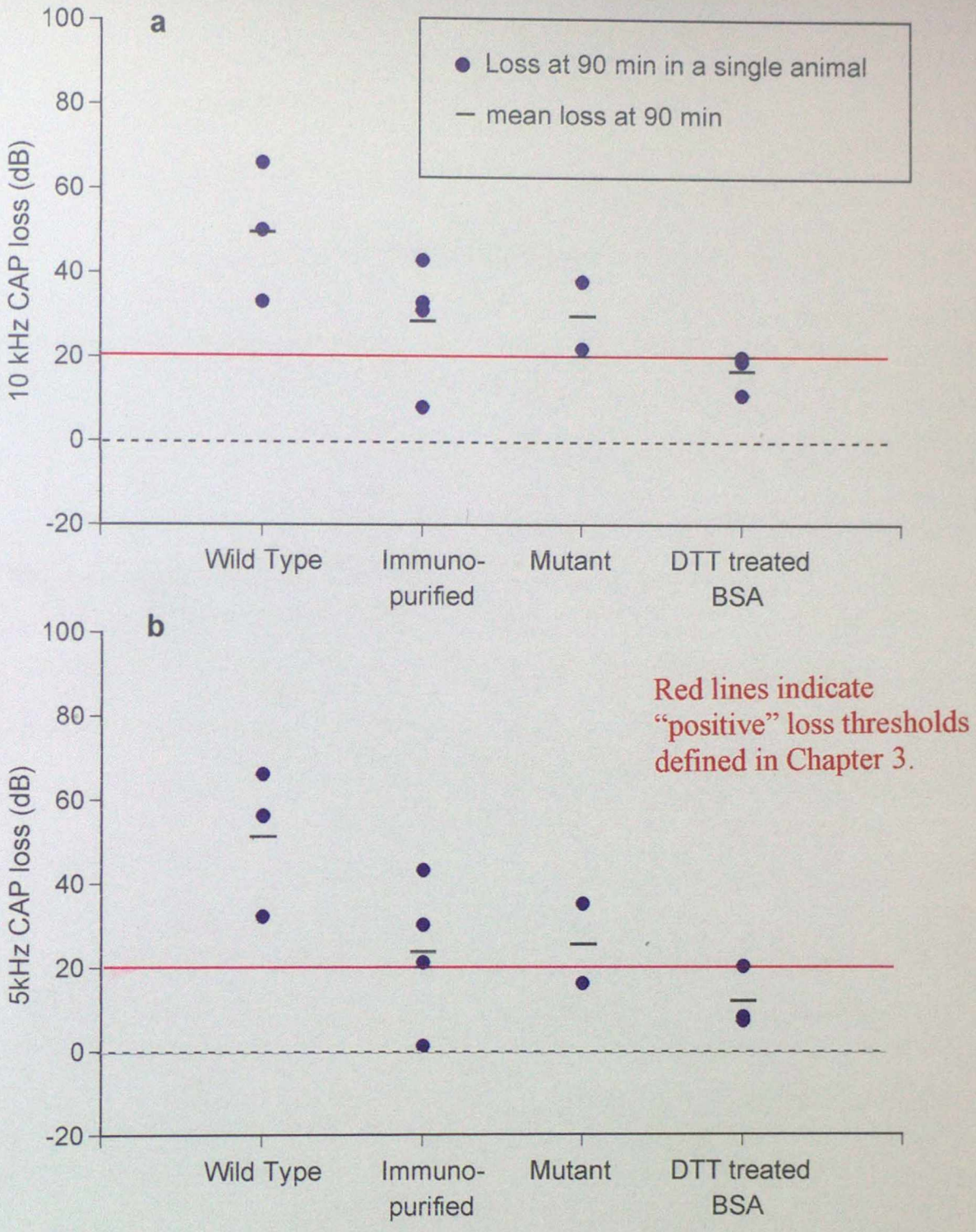


Figure 8B 3.5. 90 min losses in CAPs and CMs produced by three pneumococcal extracts (II), and DTT treated BSA.

a-b) CAP elicited with 10 and 5kHz stimuli respectively.

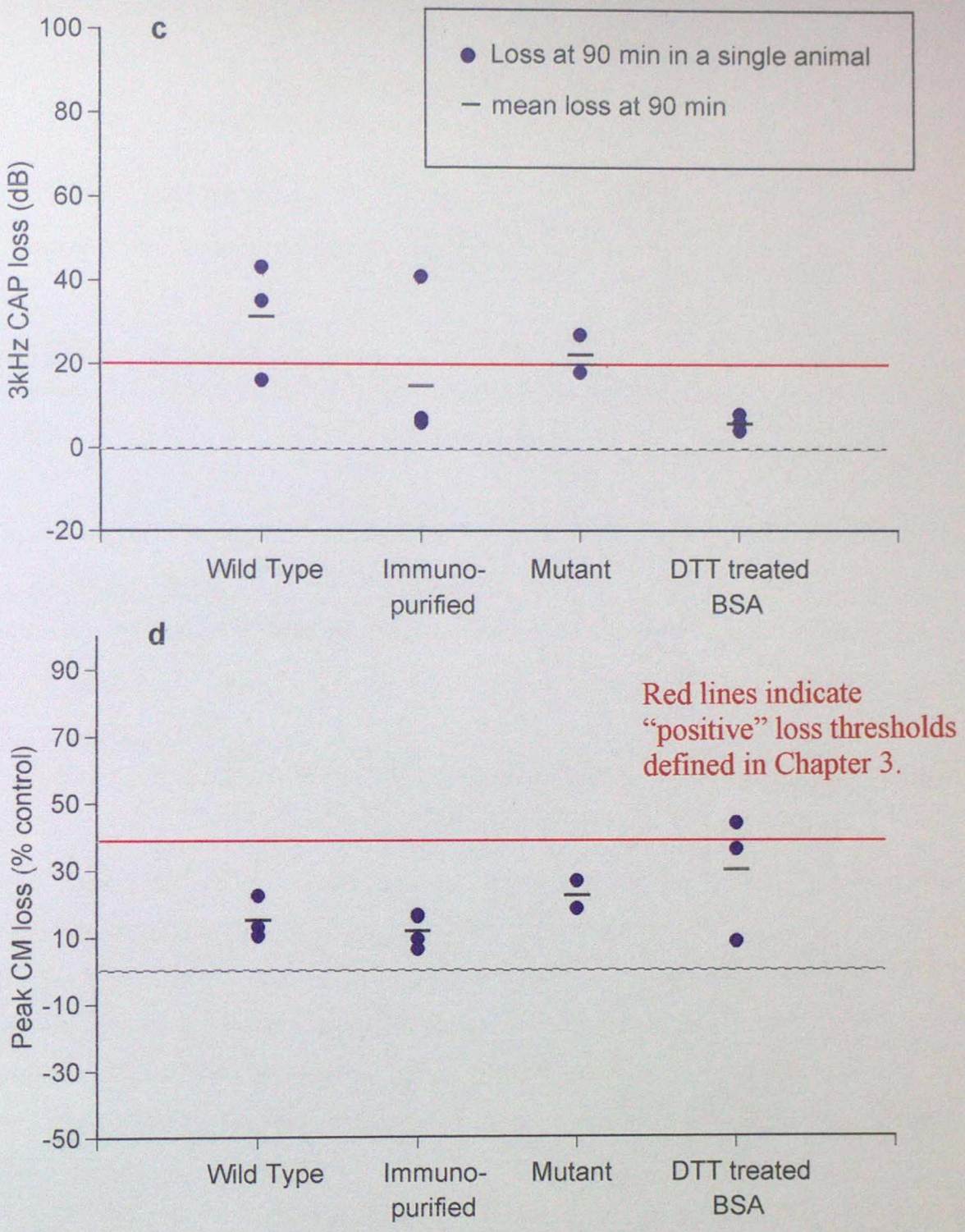


Figure 8B 3.5. continued. **90 min losses in CAPs and CMs produced by three pneumococcal extracts (II), and DTT treated BSA.**

c) CAP elicited with 3kHz stimuli. d) CM elicited with a 5kHz stimuli.

8B 4 Protein Analysis

8B 4.1 Protein Concentrations

Protein contents of each bacterial extract (II) showed:

Wild Type extract (II)	: 0.721 $mgml^{-1}$
Immunopurified Wild Type Extract	: 1.590 $mgml^{-1}$
Mutant extract (II)	: 0.535 $mgml^{-1}$

Wild type and mutant pneumococcal extracts (II) contained comparable concentrations of protein. However, the immunopurified wild type pneumococcal extract (II) contained more than twice as much protein as the wild type and mutant extracts (II). This was investigated as described below.

8B 4.2 SDS-PAGE Stained With Coomassie Brilliant Blue And Probed With Mab7.

The protein composition of each extract (II) was examined by electrophoresis. Pneumococcal extracts (II) were analysed by 1 dimensional SDS-PAGE on a 12% resolving gel stained with Coomassie Brilliant Blue (**Fig. 8B 4.2**). A large amount of peptide was present at 66.2kDa in the immunopurified extract (II) which was significantly greater than that seen in either the wild type or mutant extracts (II), and probably represents serum albumin. The immunopurified extract (II) also contained more of a peptide present slightly above 66.2kDa which could represent antibody fragments.

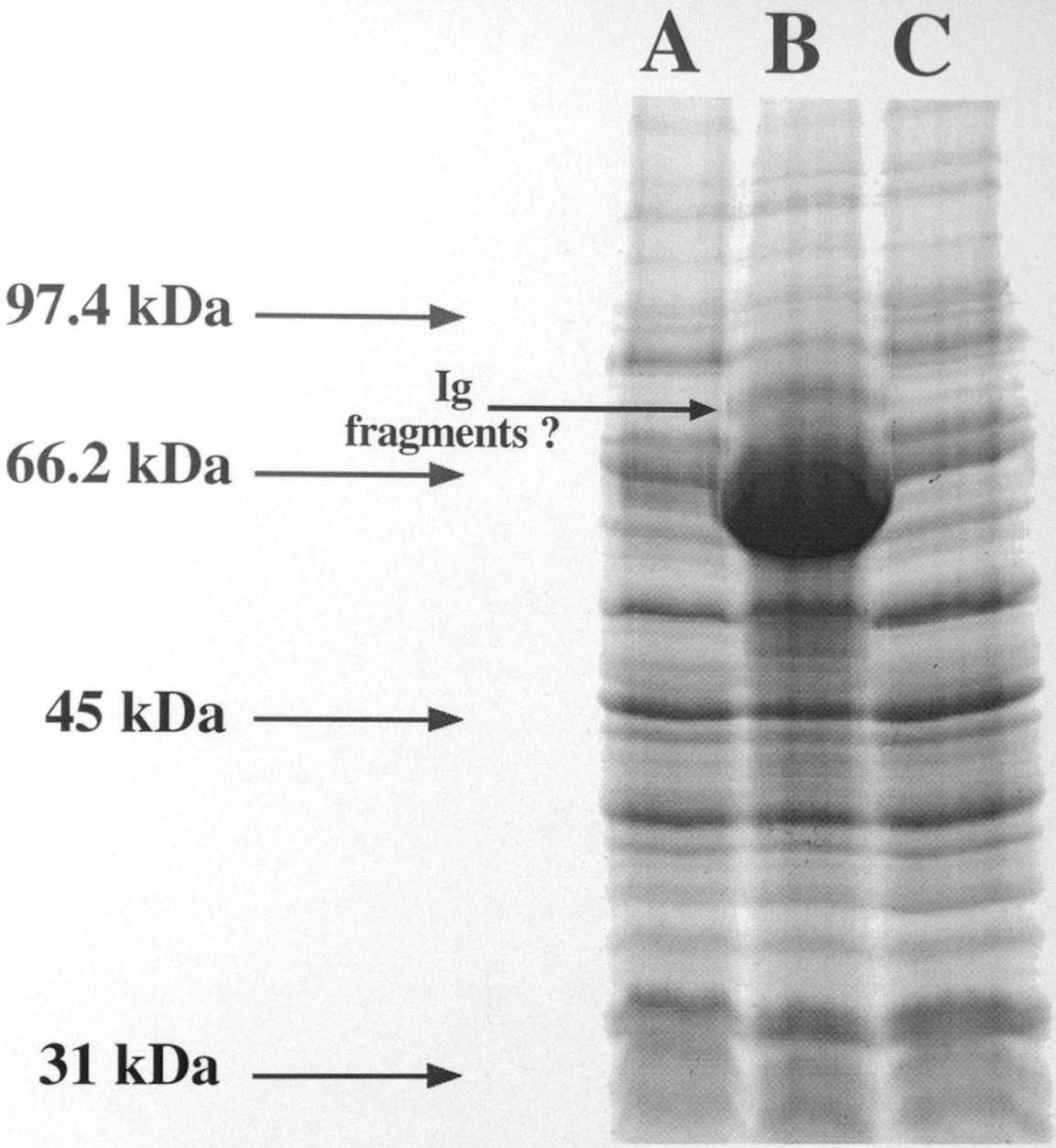


Figure 8B 4.2. **SDS-PAGE of three pneumococcal extracts (II).**

A) Wild Type Extract

B) Immunopurified Wild Type Extract

C) Mutant Extract

Conclusion

The immunopurified wild type bacterial extract (II) contained considerable amounts of protein not present in wild type and mutant extracts (II). It is suggested that two of these bands represent antibody fragments and serum albumin, probably leached from the column during immunopurification.

8B 4.3 : SDS-PAGE Probed With Monoclonal Anti-Pneumolysin Antibody (Mab7)

In order to determine whether pneumolysin was present in each of the extracts (II), they were analysed by 1 dimensional SDS-PAGE on a 12% resolving gel and probed with an anti-pneumolysin monoclonal antibody (Mab7) (**Fig. 8B 4.3**). At approximately 58kD there was a peptide present in both wild type extracts (II), but not in the mutant extract. This corresponded to pneumolysin. There was more of this peptide present in the wild type extract (II) than in the immunopurified wild type extract, based on staining intensity and comparable lane-loading of protein.

Conclusions

- Pneumolysin was present in both wild type pneumococcal extracts (II), and absent in the mutant extract. Considerably less pneumolysin was present in the wild type pneumococcal extract (II) which had been immunopurified.
- The immunopurification protocol used had removed some but not all of the pneumolysin in the wild type pneumococcal extract.

8B 4.4 : Native-PAGE Probed With Mab7

During immunopurification, anti-pneumolysin antibody may have successfully bound all pneumolysin present, but the leaching of protein from the column would have allowed some of this bound pneumolysin to contaminate the extract. In order to determine whether pneumolysin present in the immunopurified extract (II) was bound or unbound, the wild type pneumococcal

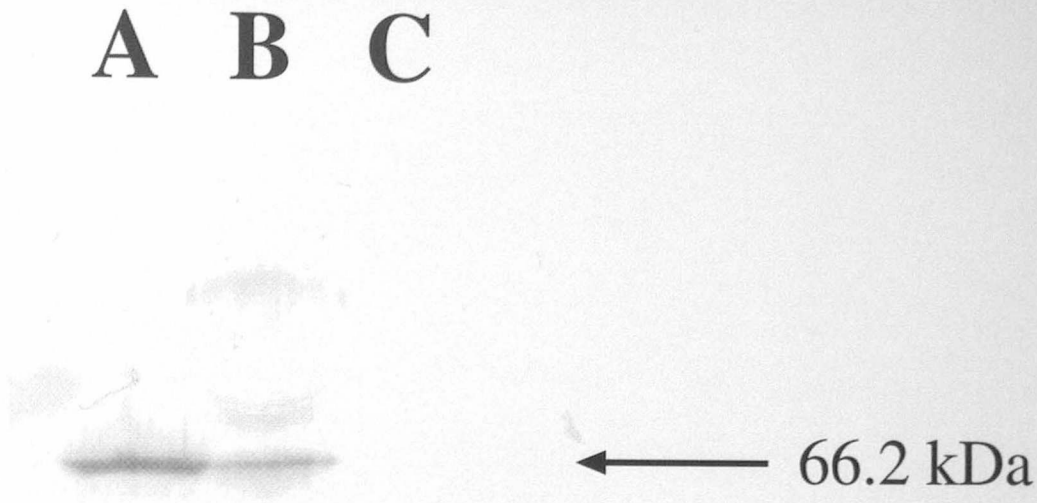


Figure 8B 4.3. **Western blot of three pneumococcal extracts (II), to analyse pneumolysin content using anti-pneumolysin monoclonal antibody.**

A) Wild Type Extract

B) Immunopurified Wild Type Extract

C) Mutant Extract

extracts (II) were analysed by 1-dimensional native-PAGE on a 5% resolving gel and probed with Mab7 (**Fig. 8B 4.4**). This method separates non denatured proteins on the basis of their size and charge density. Despite the rather poor quality of the gel, it is possible to demonstrate that pneumolysin present within the immunopurified wild type extract (II) moved only a short distance in comparison to pneumolysin in untreated pneumococcal wild type extract.

Conclusions

Pneumolysin present in immunopurified wild type pneumococcal extract (II) had a higher molecular mass than that present in wild type pneumococcal extract, suggesting that it was bound to anti-pneumolysin antibody.

8B 4.5 Protein Analysis : Conclusions

- Wild type pneumococcal bacterial extract (II) contained free pneumolysin.
- Mutant pneumococcal bacterial extract (II) contained no pneumolysin.
- Immunopurified wild type pneumococcal extract (II) contained some pneumolysin; however it was bound to anti-pneumolysin antibody.
- Immunopurified wild type pneumococcal extract (II) also contained considerable amounts of serum albumin and antibody fragments.

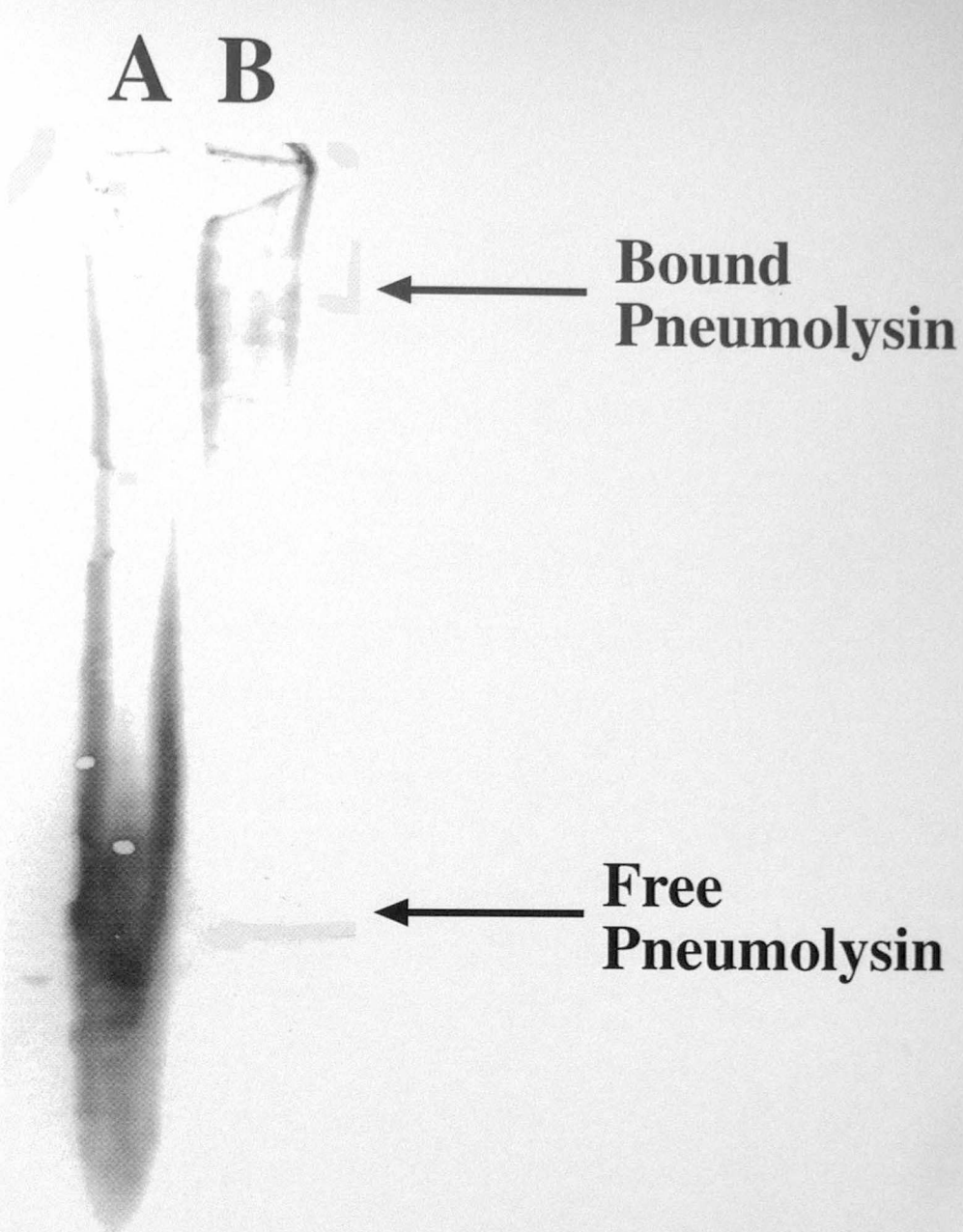


Figure 8B 4.4. Native-PAGE gel of wild type pneumococcal extracts (II), to investigate the physical state of pneumolysin using anti-pneumolysin monoclonal antibody.

A) Wild Type Extract

B) Immunopurified Wild Type Extract

The pneumolysin in lane B travelled a short distance compared to the pneumolysin in lane A, suggesting that pneumolysin in lane B is part of a high molecular weight complex.

8B 5 Ototoxicity Of Pneumococcus Type III Bacterial Extracts (II) : Overall Conclusions

- Wild type pneumococcal bacterial extract (II) contained free pneumolysin, and as would be expected, produced ototoxicity within the guinea pig cochlea.

- Mutant pneumococcal bacterial extract (II) produced ototoxicity within the guinea pig cochlea despite the absence of pneumolysin. Therefore the ototoxicity produced by this extract was due to a 'non-pneumolysin' ototoxic factor.

- Immunopurified wild type bacterial extract (II) produced some ototoxicity in the guinea pig cochlea despite the absence of **free** pneumolysin. However, immunopurified wild type bacterial extract (II) contained some antibody bound pneumolysin, and some contaminants from the immunosorbent. Since high affinity rabbit polyclonal anti-pneumolysin serum has been demonstrated to be protective in the guinea pig cochlea (unpublished data; this laboratory), it is unlikely that the contaminants are responsible for the ototoxicity produced by the immunopurified extract. As all extracts were standardised with respect to protein concentration, the bacterial contents of the immunopurified extract would have been considerably diluted in comparison to the wild type and mutant extracts. Therefore, the variable ototoxicity produced by the immunopurified extract may represent a low concentration of a 'non-pneumolysin' ototoxic factor in this extract. This in fact enhances the potential significance of the immunopurified data.

- In summary, there was evidence for an ototoxic factor in the mutant extract (II) which may represent a novel ototoxin.

- The results presented here are not consistent with previous preliminary data which suggested that ototoxicity was produced by wild type and immunopurified wild type S.pneumoniae type

III extracts, but **not** by 'non-pneumolysin producing' S.pneumoniae type III extracts (unpublished data Comis 1992). The lack of DTT treatment in previous experiments, could have been responsible for the lack of activity of the mutant extract. **If** this were the case it would imply that the novel ototoxic factor is thiol-activated. Alternatively, since the protein concentration of the pneumococcal extracts was not reported in the previous study, the concentration of an ototoxic factor in that extract may have been insufficient to produce ototoxicity. Either explanation is supported by the data from perfusions of 'non-pneumolysin producing' mutant extract from batch I which was neither concentrated nor DTT-treated and was less consistent at producing ototoxicity than the concentrated and DTT-treated extract.

CHAPTER 9

Characteristics Of Ototoxicity In Pneumococcus Type III

9.1 Electrophysiological Observations

A summary of the ototoxicity produced by batch II pneumococcal extracts is described below.

9.1.1 Wild Type Pneumococcal Extract

Perfusion of wild type pneumococcal bacterial extract (II) produced mean losses at 90 min of 50, 51 and 31 dB in the 10, 5 and 3 kHz CAP respectively, and 15% in the CM. CAP losses occurred throughout the 90 min, however the rate of decline was greatest between 20 and 60 min. Greatest CM loss occurred between 60 and 90 min however losses in the CM did not reach significance in any animal. Similar CAP losses were seen at 10 and 5 kHz, however the 3kHz CAP losses were considerably smaller.

9.1.2 Immunopurified Wild Type Pneumococcal Extract

Perfusion of immunopurified wild type pneumococcal extract (II) produced mean losses at 90 min of 29, 24 and 15 dB in the 10, 5 and 3 kHz CAP respectively, and 12% in the CM. The rate of decline in the CAP was greatest between 40 and 90 min. Greatest CM loss occurred between 30 and 50 min, however the losses did not reach positive levels in any animal. Similar CAP losses were seen at 10 and 5kHz, however the 3kHz CAP losses were considerably smaller.

9.1.3 Mutant Pneumococcal Extract

Perfusion of mutant pneumococcal extract (II) produced mean losses at 90 min of 30, 25 and 22 dB in the 10, 5 and 3 kHz CAP respectively, and 22% in the CM. The rate of decline in the CAP varied between animals and between frequencies. Animal 19 achieved most of its CAP loss within the first 20 min. Animal 20 showed similar timing in CAP loss at 3kHz, but a more gradual loss in the 10 and 5 kHz CAPs in which greatest losses were between 60 and 90 min. Greatest CM losses

were seen between 30 and 60 min, however the losses did not reach positive levels in either animal. Similar CAP losses were seen at all frequencies.

9.1.4 Electrophysiological Observations: Conclusions

- Ototoxicity produced by all pneumococcal extracts (II) involved CAP losses despite no loss in the CM. Therefore the ototoxicity was due to an effect on either the inner hair cells, afferent synapses and/or afferent neurons, whilst the outer hair cells were relatively unaffected.
- • All extracts (II) produced some positive CAP loss at all frequencies tested, therefore the ototoxicity produced by each extract (II) affected a considerable portion of the cochlea. In some extracts (II), loss in the 3kHz CAP was considerably less than seen in the 10 and 5kHz CAP, this may be due to the greater number of contributing fibres to the CAP at this frequency and hence loss of activity in a small number of fibres would have little affect on the CAP. . In addition smaller CAP losses displayed at 3kHz may have been a result poor penetration of the extract to the apex of the cochlea.
- The ototoxicity produced by extracts (II) which did not contain free pneumolysin more often involved greatest losses in the CAP in the later half of the experiment, whereas wild type extract (II) containing free pneumolysin produced greatest losses in the first half of the experiment. This may represent a difference in the rate of intoxication of the two ototoxins. Pneumolysin produces greatest losses in the first 10 min following perfusion (Comis *et al.* 1993). The potential 'novel pneumococcal ototoxin' may produce ototoxicity which is slower in action and therefore responsible for later CAP losses as displayed in animals treated with extracts (II) with free pneumolysin absent.
- The results presented here are not consistent with previous preliminary studies in which the ototoxicity produced by both unpurified (pneumolysin containing) and purified wild type pneumococcal extracts was similar (as judged by CAP losses) and was relatively slow in onset (majority of the CAP loss after 20-30min). This difference is unlikely to due to concentration differences, because even at low concentrations pneumolysin-mediated effects are fast (Comis *et al.* 1993). However, it could have been because the wild type extracts used previously were

not DTT-treated and so pneumolysin present in the unpurified extract may have been inactivated by oxidation.

9.2 Morphological Observations

9.2.1 Wild Type Pneumococcal Extract

Scanning Electron Microscopy (SEM) examination of the cochleae treated with wild type pneumococcal extracts (II) (animals 9-13) was very difficult to achieve because the cochleae were damaged during the preparation for examination. Treated cochleae were more readily damaged than control (right) cochleae which had undergone identical preparation. Therefore it could be argued that the pneumococcal extract contributed to this damage. In an attempt to lessen the damage to the cochlea by this extract, and thus enable examination by SEM, animal 7 was killed at 30 min. Examination of this cochlea by SEM was achieved and revealed several types of damage. Animal 7 displayed high initial auditory thresholds, which may indicate some pre-existing cochlear damage. However, the right cochlea displayed no damage, and the damage seen was characteristic of that associated with pneumolysin-mediated ototoxicity. Therefore it is likely that the damage seen is representative of damage evoked by perfusion of the extract (II) rather than pre-existing damage. Large craters were present in the supporting cells between outer hair cells of all rows **(Plate 9i)**. Large swellings were also seen in these supporting cells which may well represent a prerequisite to crater formation **(Plate 9ii)**. Similar swelling was seen in some inner hair cells. There was evidence of damage to stereocilia of inner hair cells and outer hair cells in all three rows. Stereocilial damage included breakage of linkages between stereocilia and collapse of stereocilia **(Plates 9iii & iv)**.

Transmission Electron Microscopy (TEM) examination of cochleae from animals treated with neat wild type pneumococcal bacterial extract (II) revealed a specific pattern of damage. The most striking and consistent damage was seen to the mitochondria of the nerve terminals at the base of the inner hair cells and in the base of the outer hair cells. Mitochondria were swollen, empty or ruptured. In addition, membranes of the nerve terminals at the base of the inner hair cells were damaged, and the cytoplasmic contents of the outer hair cells were thin or absent in patches.

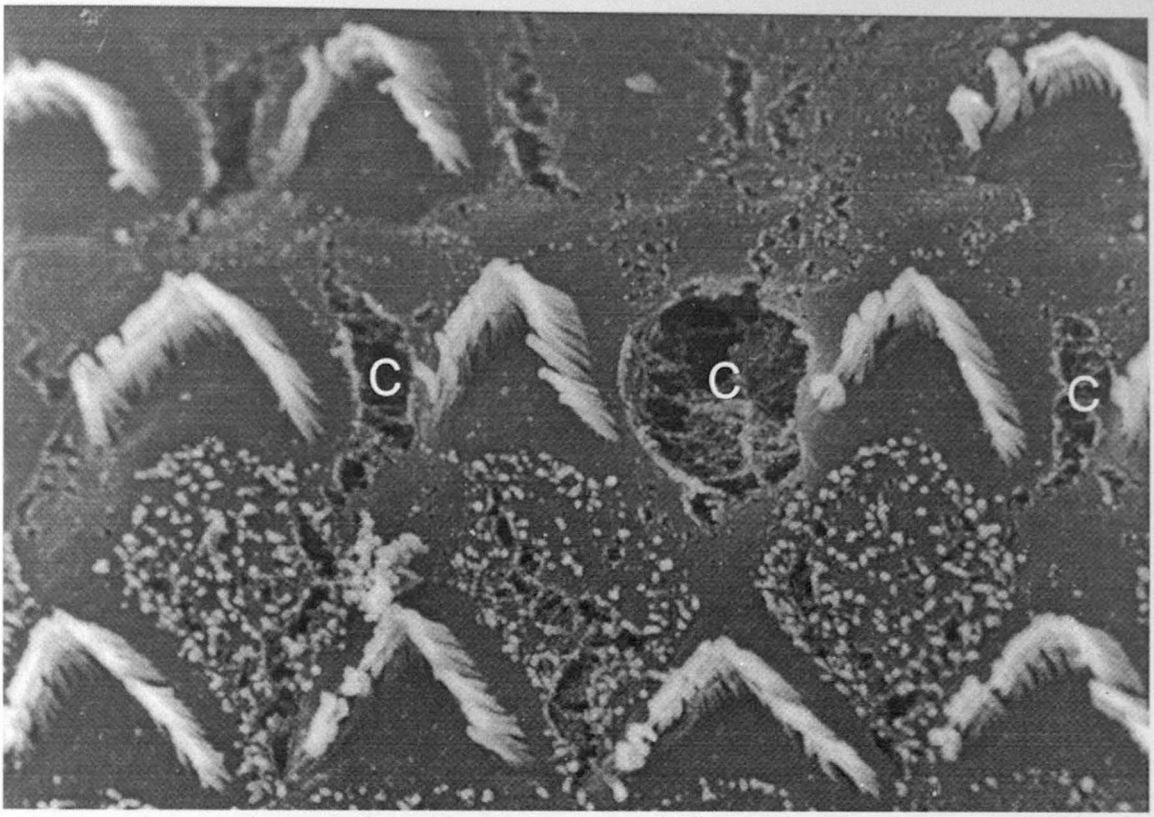


Plate 9i. **High power SEM of the OHCs and supporting cells of the left cochlea from animal 7 treated with neat wild type pneumococcal extract (II). Large craters are seen in the supporting cells between outer hair cells (C).** Magnification x 4500.

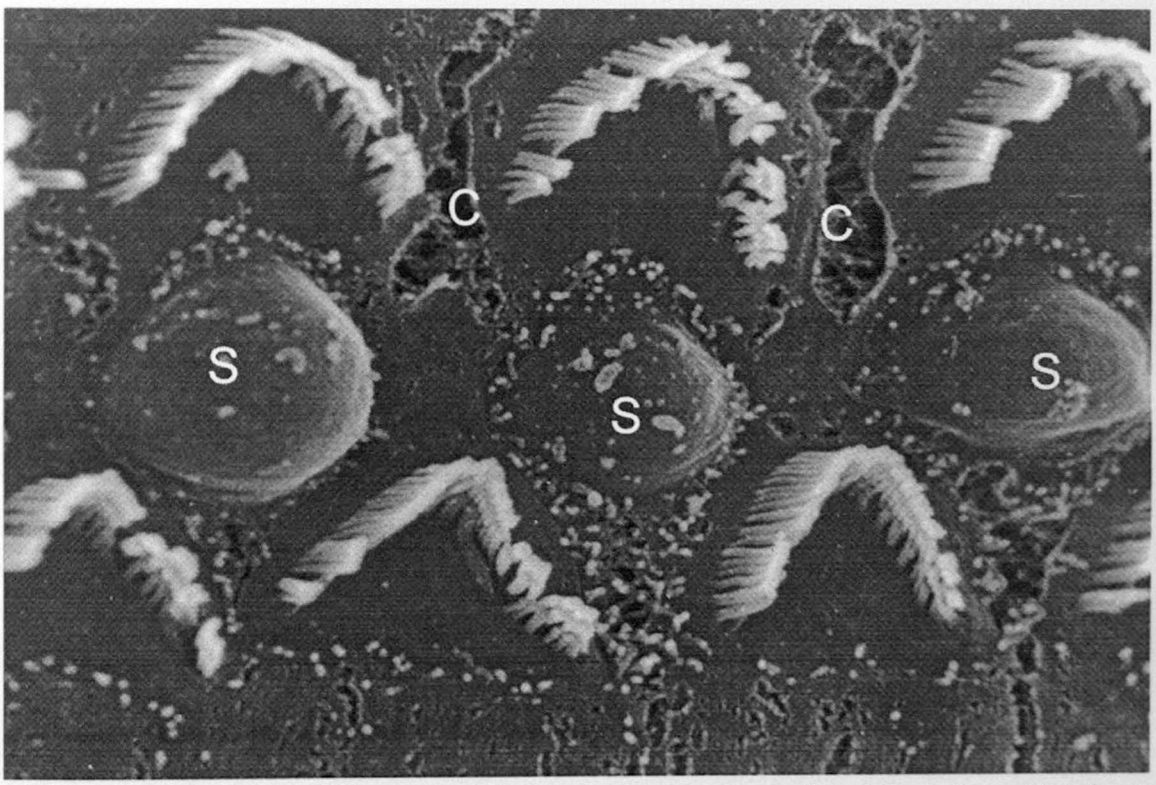


Plate 9ii. **High power SEM of the OHCs and supporting cells of the left cochlea from animal 7 treated with neat wild type pneumococcal extract (II). Large swellings are seen in the supporting cells between outer hair cells (S), as well as cratering between adjacent outer hair cells (C).** Magnification x 6000.

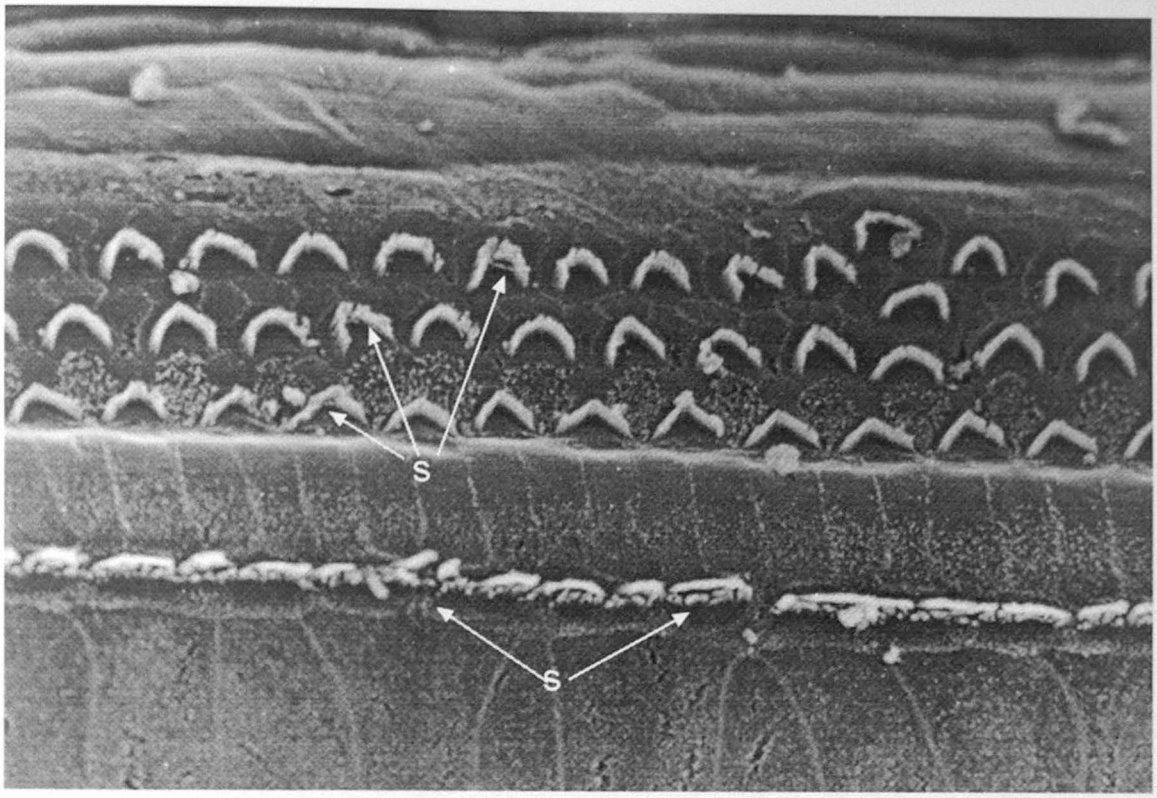


Plate 9iii. Low power SEM of the left cochlea from animal 7 treated with neat wild type pneumococcal extract (II) showing some splaying (s) of the stereocilia of IHCs and some OHCs of all 3 rows. Magnification x 1200.

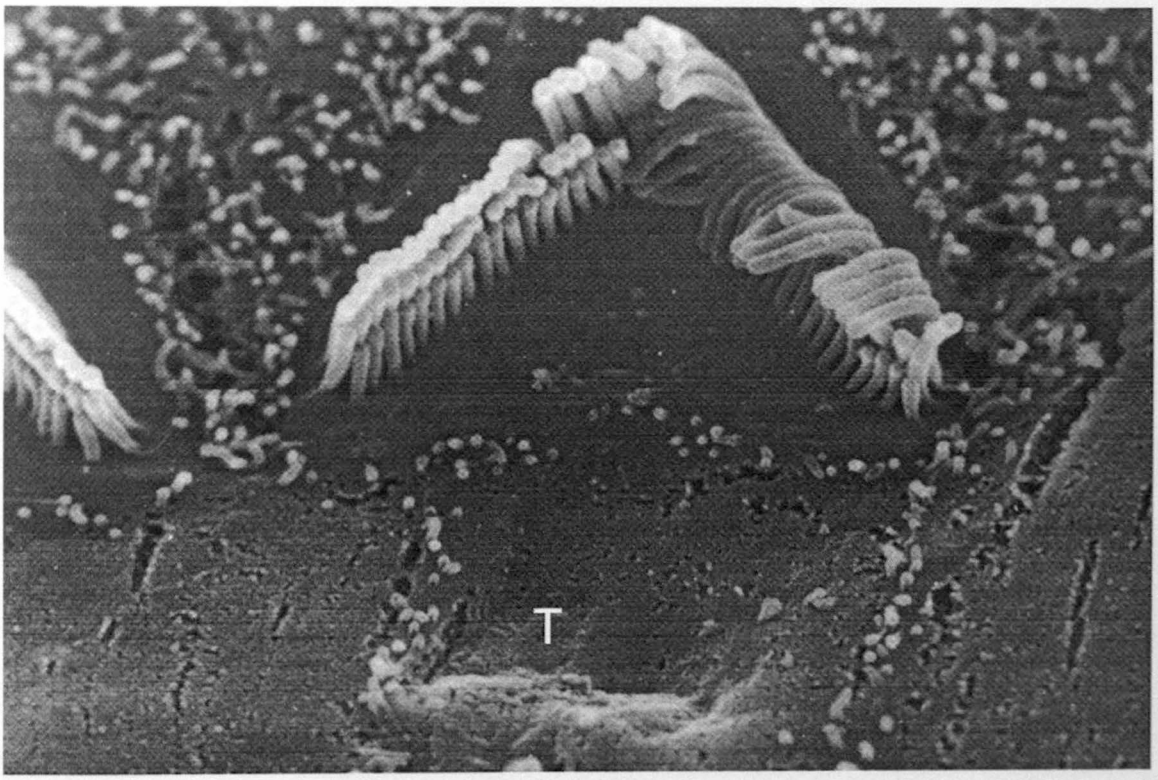


Plate 9iv. High power SEM of an OHC from the left cochlea of animal 7 treated with neat wild type pneumococcal extract (II). Some stereocilia have lost their rigidity and collapsed, and there is a tear (T) in the apical surface of the adjacent supporting cell.

Magnification x 12000.

9.2.2 Immunopurified Wild Type Pneumococcal Extract

SEM examination of cochleae treated with immunopurified wild type pneumococcal bacterial extract (II) revealed some damage to the organ of Corti, however this damage was neither consistent between animals, nor consistent with CAP losses. In animal 11 there was some damage to the inner hair cells at the base of the cochlea. The stereocilia of these inner hair cells were often splayed or detached and in some cases showed a thinning in diameter at their base (**Plate 9v**). In addition many of the adjacent supporting cells contained craters. There was also some evidence of damage to the outer hair cells at the apical end of the organ of Corti which involved splaying of the stereocilia and tearing of the adjacent supporting cell surface. Cochleae from animals 12 and 13 revealed no morphological damage.

9.2.3 'Non-pneumolysin producing' Mutant Pneumococcal Extract

SEM examination of cochleae treated with mutant bacterial extract (II) revealed some damage in animal 16, but not in animal 15. In animal 16, there was some patchy damage to the stereocilia of inner hair cells and outer hair cells of all three rows. Stereocilia were often splayed, fused together, and/or detached (**Plate 9vi**).

9.2.4 DTT-treated BSA

SEM examination of cochleae treated with DTT-treated BSA revealed no damage to the organ of Corti.

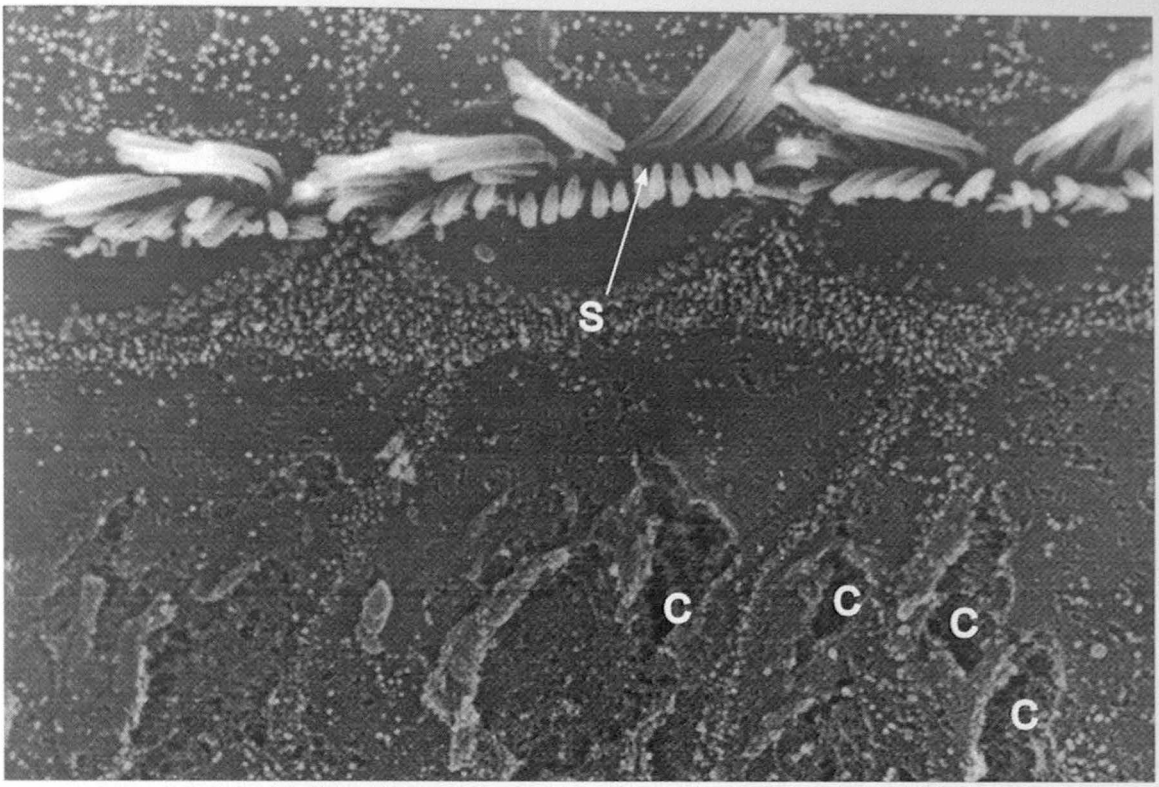


Plate 9v. High power SEM of IHCs from the left cochlea of animal 11 treated with immunopurified pneumococcal extract (II). Stereocilia of the IHCs are splayed (S) and are thinner at their base, and there are craters in the supporting cells (C).
Magnification x 6000

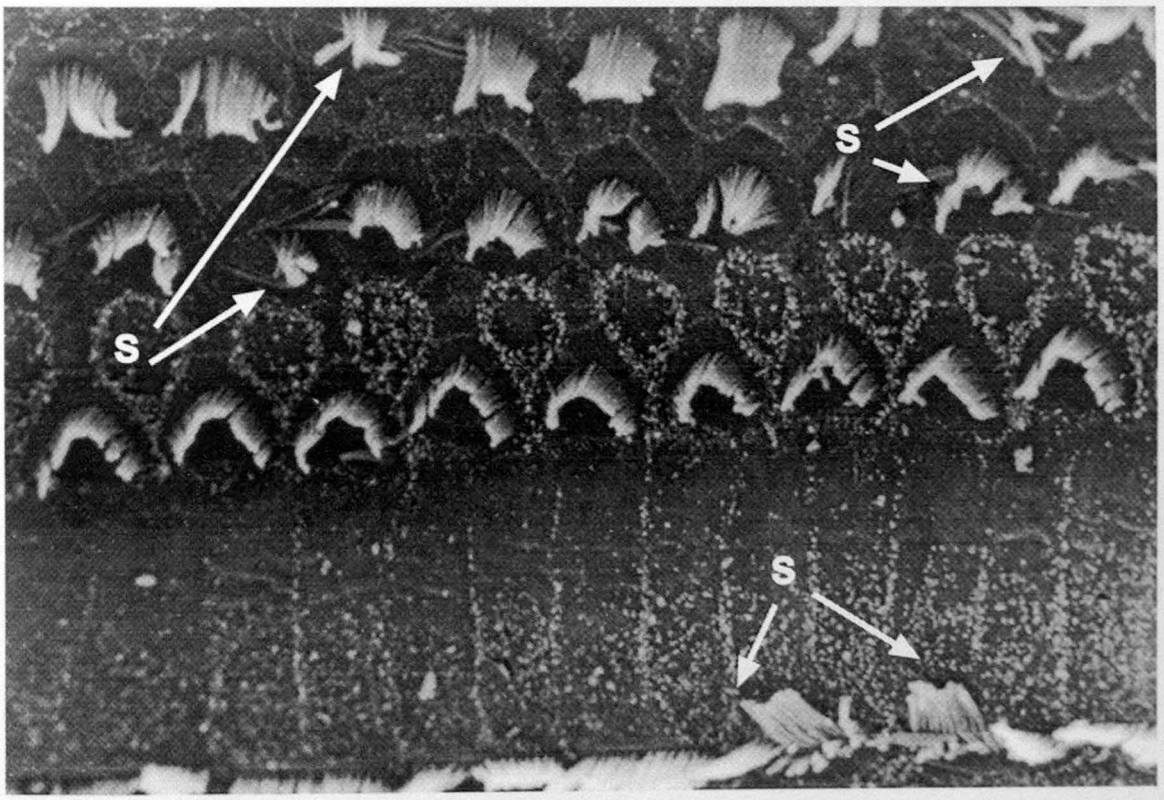


Plate 9vi. Low power SEM of the left cochlea from animal 16 treated with mutant pneumococcal extract (II) showing splaying (s) of the stereocilia of IHCs and OHCs of rows 2 and 3. Magnification x 2250

9.2.5 Morphological Data: Conclusions

From the limited data tentative conclusions can be drawn concerning the morphological damage produced by each extract.

- Wild type pneumococcal bacterial extract (II) produced some swelling and cratering in the supporting cells of the organ of Corti along with splaying and detachment of stereocilia of inner and outer hair cells. However this result is from one animal.
- Immunopurified wild type pneumococcal bacterial extract (II) produced some cratering to supporting cells and splaying or detachment of stereocilia similar to that seen in cochleae treated with the unpurified wild type extract. However the results were not consistent between animals.
- Mutant bacterial extract (II) produced some patchy damage which was limited to stereocilia. Splaying, detachment and fusing of stereocilia of outer and inner hair cells was observed. However the results were not consistent between animals.
- In summary, the wild type extract (II) may have produced severe damage to the organ of Corti which prevented examination by SEM. The kind of damage that this extract (II) produced probably involved cratering of supporting cells which led to the widespread damage during SEM preparation. Immunopurified extract (II) produced moderate damage to the organ of Corti similar to that seen in wild type extract (II) treated cochlea after 30 min. Mutant extract (II) produced some damage to the organ of Corti, but considerably less severe than that observed in both purified and non purified wild type extracts (II) .
- TEM examination of cochleae treated with wild type pneumococcal extract revealed striking damage to the mitochondria of the nerve endings to the inner hair cells, and in the base of the outer hair cells. Therefore, cochleae treated with immunopurified and mutant extracts which revealed no morphological damage by SEM, despite positive CAP losses, may have similar underlying mitochondrial damage which cannot be seen using the SEM technique.

CHAPTER 10

Excitotoxicity Within The Cochlea

Excitatory amino acids, nitric oxide (NO) and reactive oxygen species (ROSs) are all ototoxic in the cochlea (Eybalin 1993; Amaee *et al.* 1997 and Clerici and Yang 1996), and have been implicated in the deleterious outcomes that are associated with bacterial meningitis (reviewed in Pfister *et al.* 1994 and Miltstien, Sakai, Brew *et al.* 1994). Therefore they may be involved in meningogenic hearing losses. The relationship between the ototoxicity produced by all three is not clear. We have recently reported that either inhibition of nitric oxide synthase (NOS), or scavenging for superoxide considerably attenuates the excitotoxicity evoked by perfusion of N-methyl-D-aspartate (NMDA) into cochleae (Amaee *et al.* 1997). Therefore it seems likely that excitotoxicity mediated by NMDA receptors within the cochlea is at least in part mediated by NO and superoxide (a ROS). Despite reports of NO and ROSs as mediators of excitotoxicity produced by non-NMDA receptors in brain and retinal cells (Dutrait *et al.* 1995 and Schulz, Matthews, Jenkins *et al.* 1995), and the predominant role played by non-NMDA receptors in the evolution of excitatory damage, there is at present no experimental data to support or reject a similar relationship between NO or ROSs and non-NMDA mediated excitotoxicity in the cochlea. Therefore, the present study was carried out in order to investigate the potential role of NO and ROSs in the excitotoxicity mediated by non-NMDA receptors within the cochlea. Excitotoxicity was assessed by electrophysiological recordings and morphological examinations. To my knowledge this is the first work of this kind.

A total of 46 animals were used in the work described in this chapter; the initial auditory thresholds of these animals can be seen in **Fig. 10**. Five animals were excluded from statistical and summary data as they displayed initial auditory thresholds above the defined level. Animals were killed at several different time points in order to examine cochleae at various stages of excitotoxicity. However, for the purpose of statistical comparisons, all electrophysiological losses were compared at 30 min.

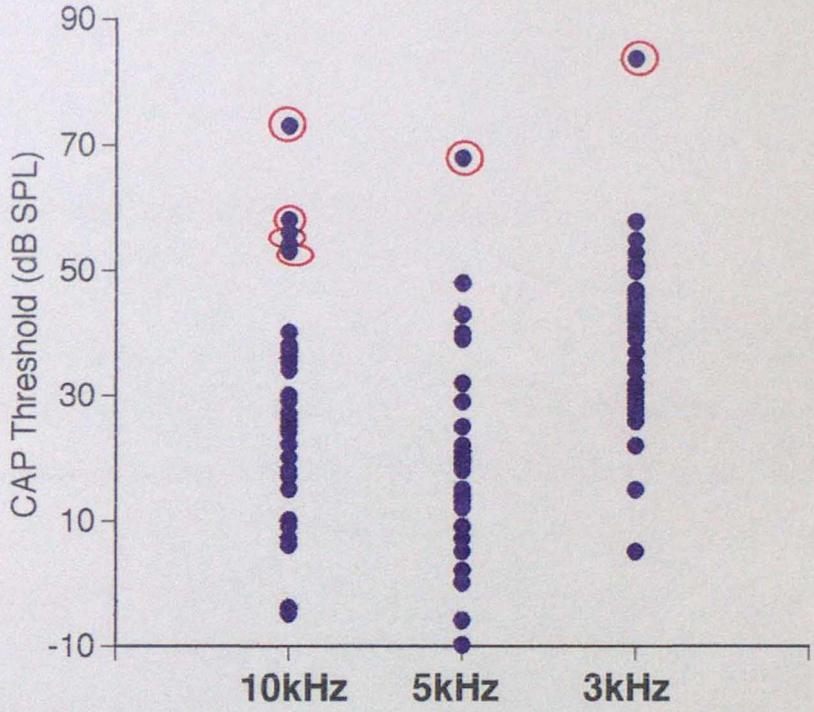


Figure 10. Threshold CAP values of all animals used in the work described in this chapter.

Red circles indicate thresholds above the defined level
(50dB at 10 and 5kHz and 65dB at 3kHz),
or well outside the normal distribution of the group.

Aims and objectives

1. To perfuse non-NMDA agonists quisqualate and kainate into scala tympani of guinea pig cochleae.
2. To pre-treat cochleae with nitric oxide inhibitors, or free radical scavengers prior to perfusion of quisqualate or kainate.
3. To measure electrophysiological recordings of the CAP and CM.
4. To carry out morphological investigations of treated cochleae.

10.1 Excitotoxicity Produced By Kainate and Quisqualate Alone

In order to establish the degree of excitotoxicity produced by excessive stimulation of the non-NMDA receptors, kainate (a selective agonist for kainate receptors) and quisqualate (a selective agonist for α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and metabotropic receptors) were tested for excitotoxicity in cochleae which had not been pre-treated. Although quisqualate does not discriminate between AMPA and metabotropic receptors, it was felt that the most rapid and *least costly* approach would be to investigate excitotoxicity produced by quisqualate first, and then to proceed to more selective agonists (e.g. AMPA) if the results were found to be positive. Previous reports of electrophysiological responses in guinea pig cochleae to cumulative doses of these agonists demonstrate a steep slope in the electrophysiological responses to increasing doses of kainate and quisqualate (Bledsoe, Bobbin and Chihal 1981; Jenison, Winbery and Bobbin 1986). Since these reports were from cumulative doses of both agonists, the electrophysiological responses may have been enhanced, particularly in the case of kainate because of its inability to produce desensitisation in other cells (Kiskin, Krishtal and Tsyndrenko 1986). Therefore supra maximal doses were used initially in order to achieve positive CAP losses (as defined in Chapter 3) from single perfusions.

10.1.1 10mM Kainate

10mM kainate was perfused into cochleae of animals 1 and 2 **(Table 10.1.1; Fig.10.1.1)** and produced large CAP losses at all frequencies in both animals. At 30 min, 10kHz CAP losses were 68 and 89 dB in animals 1 and 2 respectively. Positive CM losses were not displayed by either animal.

Table 10.1.1 : Electrophysiological losses evoked by perfusion with 10mM kainate in two animals. Positive losses are bold.

Time post perfusion (min)	Animal 1				Animal 2			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	20	13	41	0.13	-4	-10	36	0.23
	Loss in CAPs (dB) or CM (% control)							
1	**72**	**72**	**64**	17	**83**	**93**	-	-9
5	**70**	**71**	**64**	17	**89**	**94**	-	-6
10	**70**	**71**	**64**	19	**89**	**93**	-	-6
30	**68**	**68**	**64**	17	**89**	**93**	-	-6

10.1.2 100μM Kainate

In an attempt to produce a less potent degree of kainate mediated excitotoxicity, 100μM kainate was perfused into cochleae of animals 3, 4, 5 and 6 **(Table 10.1.2; Fig.10.1.2)**. Data from animal 6 are included in Table 10.1.2 and Fig.10.1.2, but have been excluded from statistical and summary data as the animal displayed initial auditory thresholds above the defined level. Positive CAP losses were displayed by animals 4, 5, and 6, but not by animal 3. At 30 min, 10kHz CAP losses were 14, 22, 21 and 27 dB in animals 3, 4, 5 and 6 respectively. Positive CM losses were not displayed by any animal.

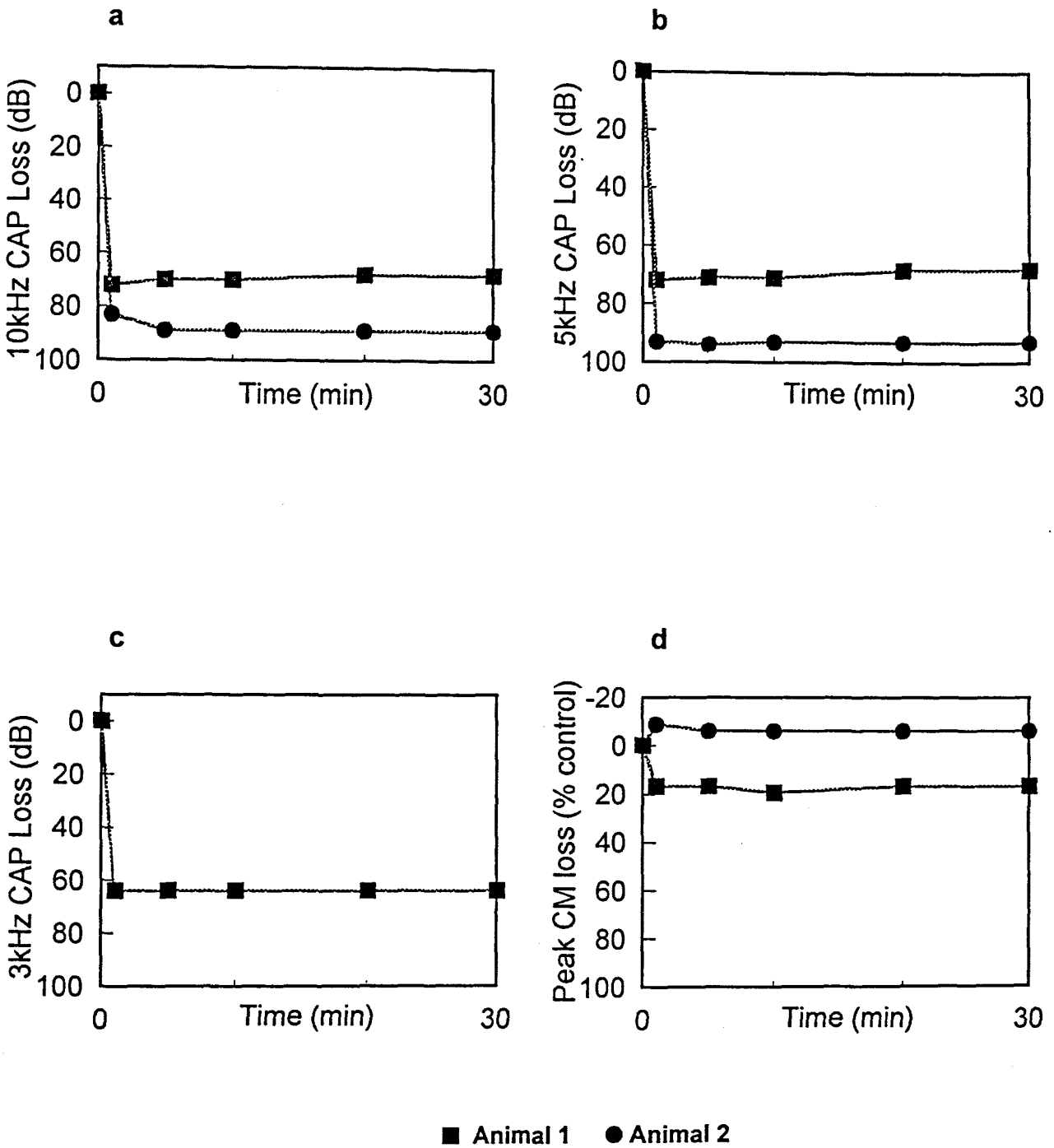


Figure 10.1.1. Losses in the CAP and CM in two animals treated with 10mM kainate.

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

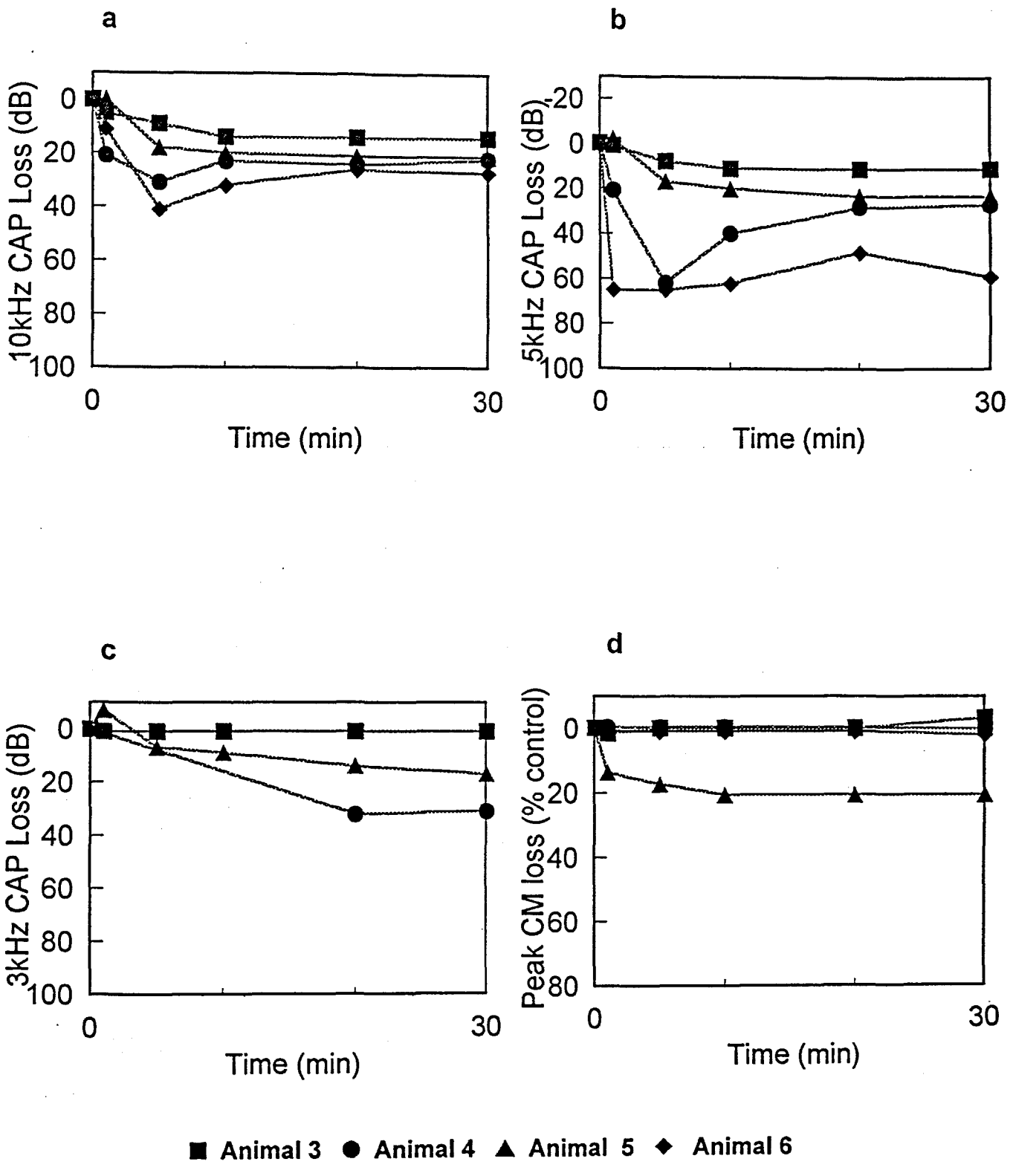


Figure 10.1.2. Losses in the CAP and CM in four animals perfused with 100μM kainate.

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

Table 10.1.2 : Electrophysiological losses evoked by perfusion with 100μM kainate in four animals. Positive losses are bold.

Time post perfusion (min)	Animal 3				Animal 4			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	18	0	29	0.12	34	20	43	0.17
	Loss in CAPs (dB) or CM (% control)							
1	5	1	1	2	**21**	**21**	-	0
5	9	8	1	0	**31**	**62**	-	0
10	14	11	1	0	**23**	**40**	-	0
30	14	11	1	-3	**22**	**27**	**31**	0

Time post perfusion (min)	Animal 5				Animal 6			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	22	2	37	0.1	54	25	47	0.23
	Loss in CAPs (dB) or CM (% control)							
1	0	-2	-7	14	11	**65**	-	1
5	18	17	7	17	**41**	**65**	-	1
10	**20**	**20**	9	21	**32**	**62**	-	1
30	**21**	**23**	17	21	**27**	**59**	-	2

10.1.3 Quisqualate

10mM quisqualate was tested for excitotoxicity in animals 7, 8, 9 and 10 (**Table 10.1.3; Fig.10.1.3**). Data from animal 7 is included in Table 10.1.3 and Fig. 10.1.3, but has been excluded from statistical and summary data as the animal displayed initial auditory thresholds outside the defined range. Positive CAP losses were displayed by all 4 animals, at all frequencies recorded. At 30 min, 10kHz CAP losses were 22, 26, 55 and 45 dB in animals 7, 8, 9 and 10 respectively. Positive CM losses were not displayed by any animal.

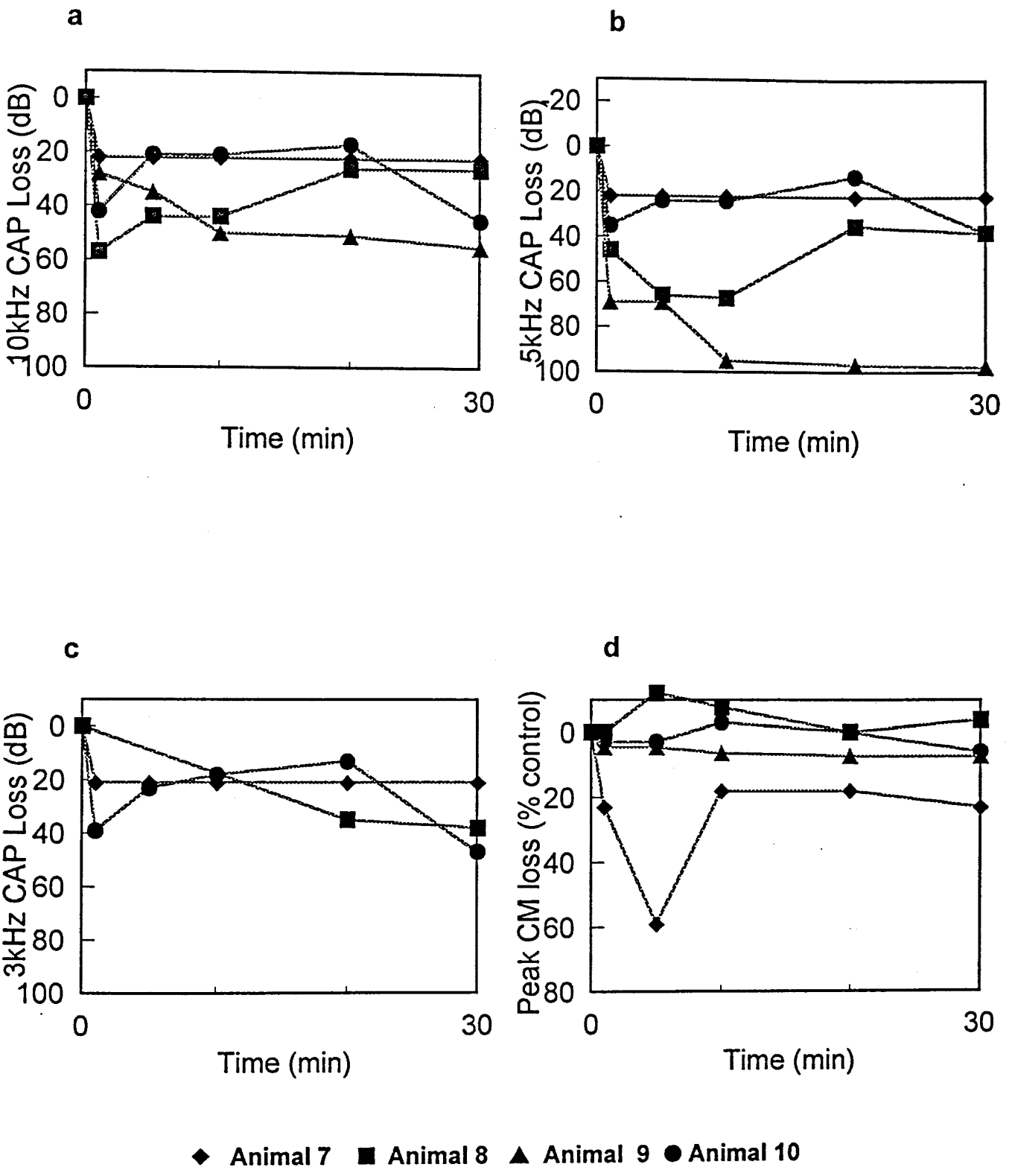


Figure 10.1.3. Losses in the CAP and CM in four animals perfused with 10mM quisqualate.

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

Table 10.1.3 : Electrophysiological losses evoked by perfusion with 10mM quisqualate in four animals. Positive losses are bold.

Time post perfusion (min)	Animal 7				Animal 8			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	73	68	84	0.06	6	15	30	0.18
	Loss in CAPs (dB) or CM (% control)							
1	**22**	**22**	**21**	23	**57**	**46**	-	0
5	**22**	**22**	**21**	**59**	**44**	**66**	-	-12
10	**22**	**22**	**21**	18	**44**	**67**	-	-8
30	**22**	**22**	**21**	23	**26**	**38**	**38**	-4

Time post perfusion (min)	Animal 9				Animal 10			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Loss in CAPs (dB) or CM (% control)							
	15	-10	28	0.22	27	19	44	0.12
	Loss in CAPs (dB) or CM (% control)							
1	**28**	**69**	-	5	**42**	**35**	**39**	3
5	**35**	**69**	-	5	**21**	**24**	**23**	3
10	**50**	**95**	-	6	**21**	**24**	18	-3
30	**55**	**98**	-	7	**45**	**38**	**47**	6

10.1.4 Excitotoxicity Produced by Kainate and Quisqualate Alone: Summary and Conclusions

Average losses in CAPs and CMs produced by perfusions of kainate and quisqualate are summarised in **Table 10.1.4.**

Table 10.1.4 : Average 30 min losses in CAPs and CMs produced by 10mM and 100μM kainate (KA), and 10mM quisqualate (QA).
Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 30 min post perfusion			
	10 kHz CAP	5 kHz CAP	3kHz CAP	CM
10mM KA (n=2)	**78 ± 15**	**80 ± 18**	**64**	5 ± 16
100μM KA (n=3)	19 ± 4	**20 ± 8**	16 ± 5	6 ± 13
10mM QA (n=3)	**42 ± 15**	**58 ± 35**	**42 ± 6**	3 ± 6

Conclusions

- 10mM kainate produced potent excitotoxicity (as judged by CAP losses >40dB) in the guinea pig cochlea. There was evidence for moderate excitotoxicity (as judged by CAP losses >20dB) produced by 100μM kainate, however this was not seen in one animal. 10mM quisqualate produced excitotoxicity in the guinea pig cochlea which was either moderate or potent (as judged by CAP losses of 20-40 or >40dB respectively).
- 10mM kainate often reduced the CAP to an extent that a threshold response (i.e. 2mV on the screen) could not be produced by the maximum stimulus intensity (95-105dB dependent on frequency see Chapter 2 for details). Therefore the calculated CAP loss was highly dependent on the initial threshold values. 10mM quisqualate and 100μM kainate did not reduce the CAP to this extent and so produced excitotoxicity which was less dependent on the size of the initial threshold values. For this reason, 100μM, rather than 10mM concentration was used to assess the degree of excitotoxicity produced by kainate in all further experiments. However, 10mM kainate was perfused on some occasions when this was deemed relevant.
- The results presented here appear to be consistent with previous studies. Jenison *et al.* (1986) reported maximal responses of 67 and 100% suppression of the CAP reached at approximately 25 and 110 μM concentrations of quisqualate and kainate respectively. This would imply that 10mM doses are supramaximal doses and should produce similar suppression. Jenison *et al.* (1986) measured suppression as the average reduced size of the CAP over a range of intensities. This cannot be compared with my data which is presented as losses in dB.

However, Bledsoe *et al.* (1981) reported a complete elimination of the 10kHz CAP at all intensities (20-100dB) by kainate at 250μM and 1mM, loss of between 50 and 60dB at 100μM, and no loss in the CAP at 1, 25 and 50μM. These results are comparable with mine in which 10mM kainate either eliminated or nearly eliminated (within 5dB) all CAPs at all intensities (-5-105dB), and 100μM kainate produced a maximum of 31dB hearing loss. The CAP losses from my data appear to be smaller, but this is likely to be because Bledsoe *et al.* (1981) used cumulative doses. Since responses to kainate in brain tissues show no desensitisation (Kiskin *et al.* 1986), this may be the case in the cochlea too which would explain greater CAP losses as a result of cumulative doses of kainate. I have found no other reports of CAP losses in response to quisqualate which are directly comparable.

10.1.5 Morphological Examinations

10.1.5.1 10mM Kainate

Cochleae treated with 10mM kainate revealed widespread damage to the base of the inner hair cell and surrounding structures. Nerve terminals at the base of the inner hair cells were absent, as was the base of the inner hair cells themselves. Outer hair cells showed very little damage, although the contents of the efferent terminals at the base of the outer hair cells were more dense than in control cochleae, and there was some membrane damage in these efferent nerve endings. There was little or no sign of mitochondrial damage **(Plates 10i & ii)**.

10.1.5.2 100μM Kainate

Cochleae treated with 100μM kainate revealed very little sign of morphological damage. There was some evidence of mitochondrial damage within afferent nerve terminals at the base of the inner hair cells, and some evidence of membrane damage at the afferent neuron-inner hair cell synapse. However, the damage was not widespread **(Plate 10iii)**.

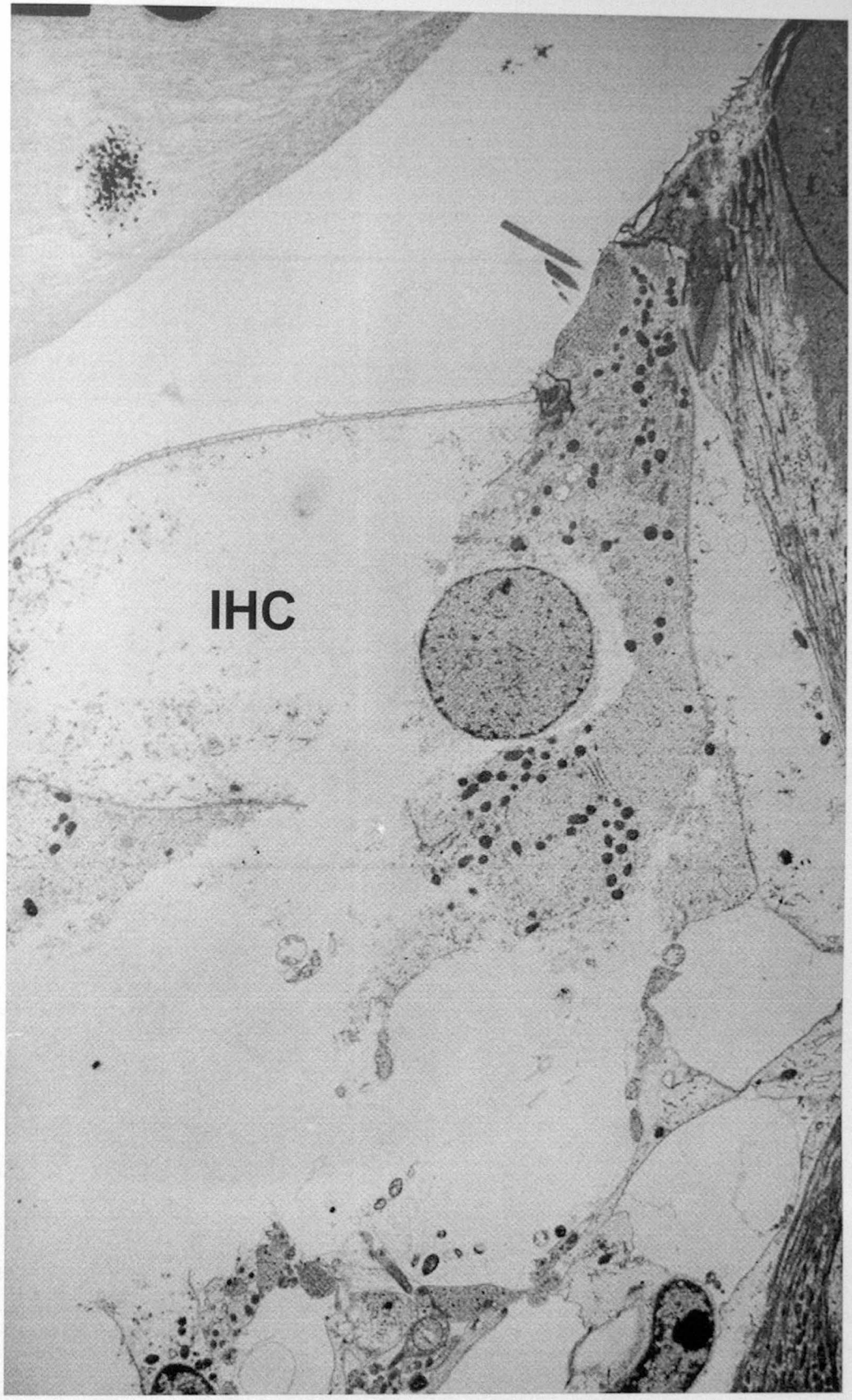


Plate 10i. Inner hair cell of the left cochlea from animal 2 treated with 10mM kainate. Extensive damage to the inner hair cell (IHC) and surrounding structures. Magnification x 4100

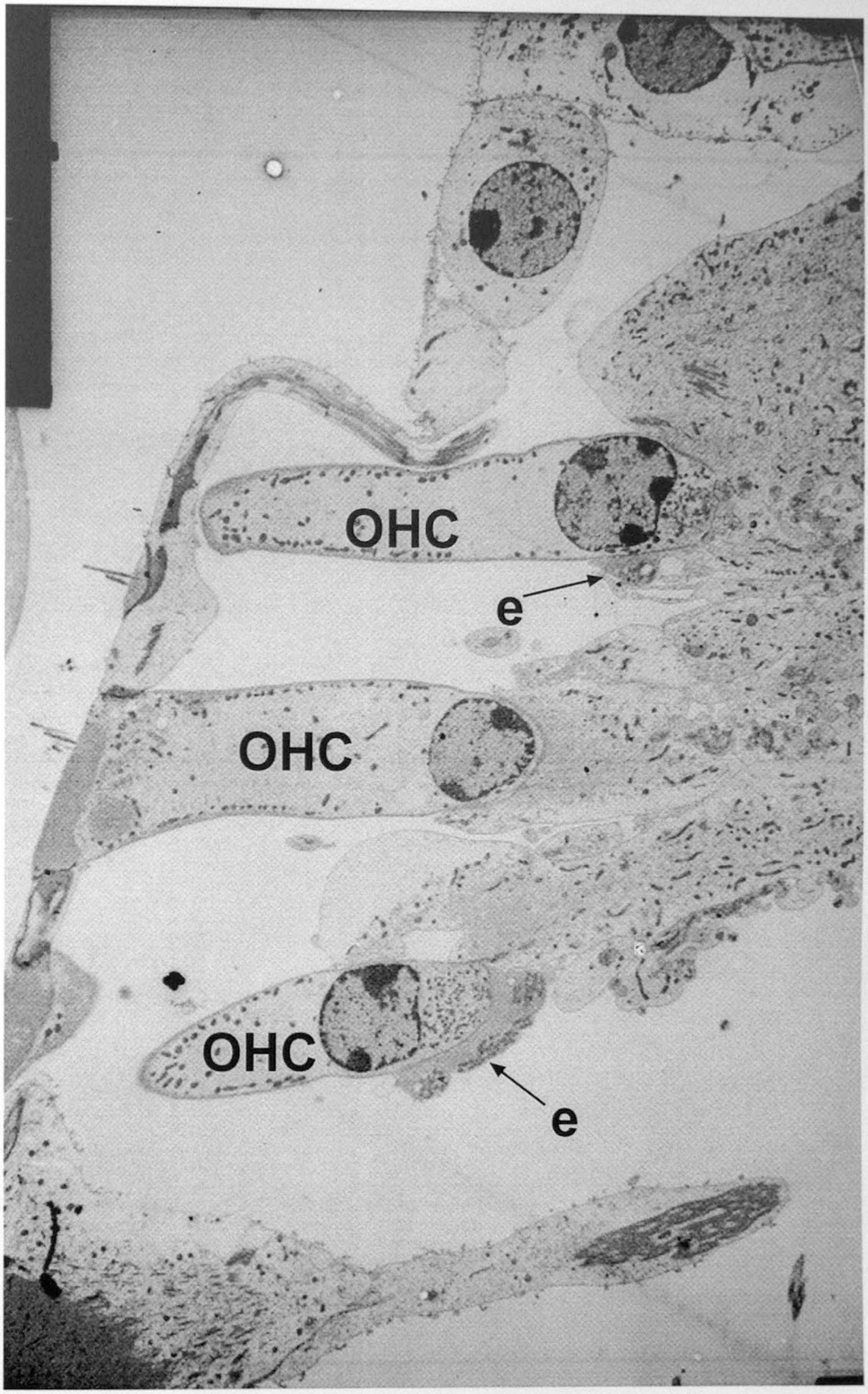


Plate 10ii. Outer hair cells of the left cochlea from animal 2 treated with 10mM kainate. Outer hair cells (OHCs) are mostly unaffected. The efferents at the base of the outer hair cells appear to be slightly more dense than in control cochleae. Magnification x 3000

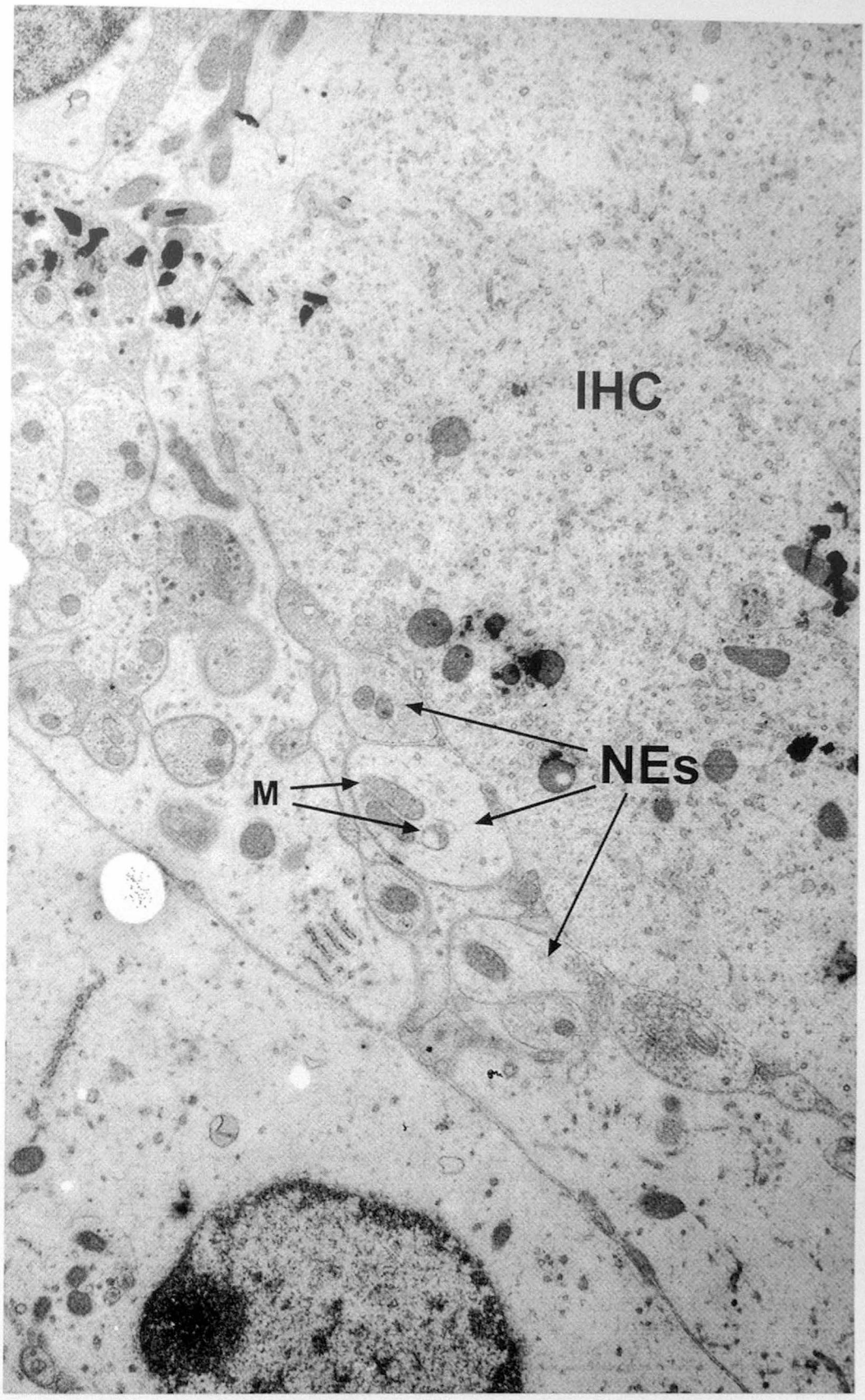


Plate 10iii. Afferent nerve endings at the base of an inner hair cell of the left cochlea from animal 4 treated with 100μM kainate. A small amount of mitochondrial (M) damage can be seen to the afferent nerve endings (NEs) at the base of the inner hair cell (IHC). Magnification x 17400

10.1.5.3 10mM Quisqualate

Cochleae treated with 10mM quisqualate revealed widespread damage to the afferent nerve terminals at the base of the inner hair cells with some associated damage to surrounding structures. The contents of the nerve terminals at the base of the inner hair cells were often absent, as was the base of the inner hair cells and supporting cells. Many mitochondria of surrounding nerve terminals were empty or swollen. Outer hair cells showed very little damage, although the contents of the efferent terminals at the base of the outer hair cells were more dense than in control cochleae and showed some indications of membrane damage **(Plates 10iv-vi)**.

10.1.5.4 Morphological Examinations : Conclusions

- 10mM kainate and 10mM quisqualate produced qualitatively similar morphological damage which was specific to the afferent nerve terminals of the inner hair cells. However, kainate produced more extensive damage than quisqualate. 100μM kainate produced very little morphological damage which was limited to mitochondrial and membrane damage at the base of the inner hair cells. The degree of morphological damage was consistent with the extent of CAP losses.
- Therefore, it appears that the primary target of excitotoxicity produced by both these agonists is the afferent nerve terminals of the inner hair cells, which then extends to surrounding structures, most likely by release of their contents. Since 100μM kainate revealed only mitochondrial and membrane damage, this may represent the early stages in afferent nerve terminal damage.
- These results are consistent with previous data concerning kainate excitotoxicity. Morphological examinations by Pujol, Lenow, Roberts *et al.* (1985) following perfusion of 500μM kainate revealed swelling of most afferent nerve terminals at the base of the inner hair cells without any damage to the hair cells themselves. Their dose response was consistent with my data since morphological damage was less extensive than that produced by 10mM kainate in this study, but more extensive than that presented here following perfusion of 100μM kainate.

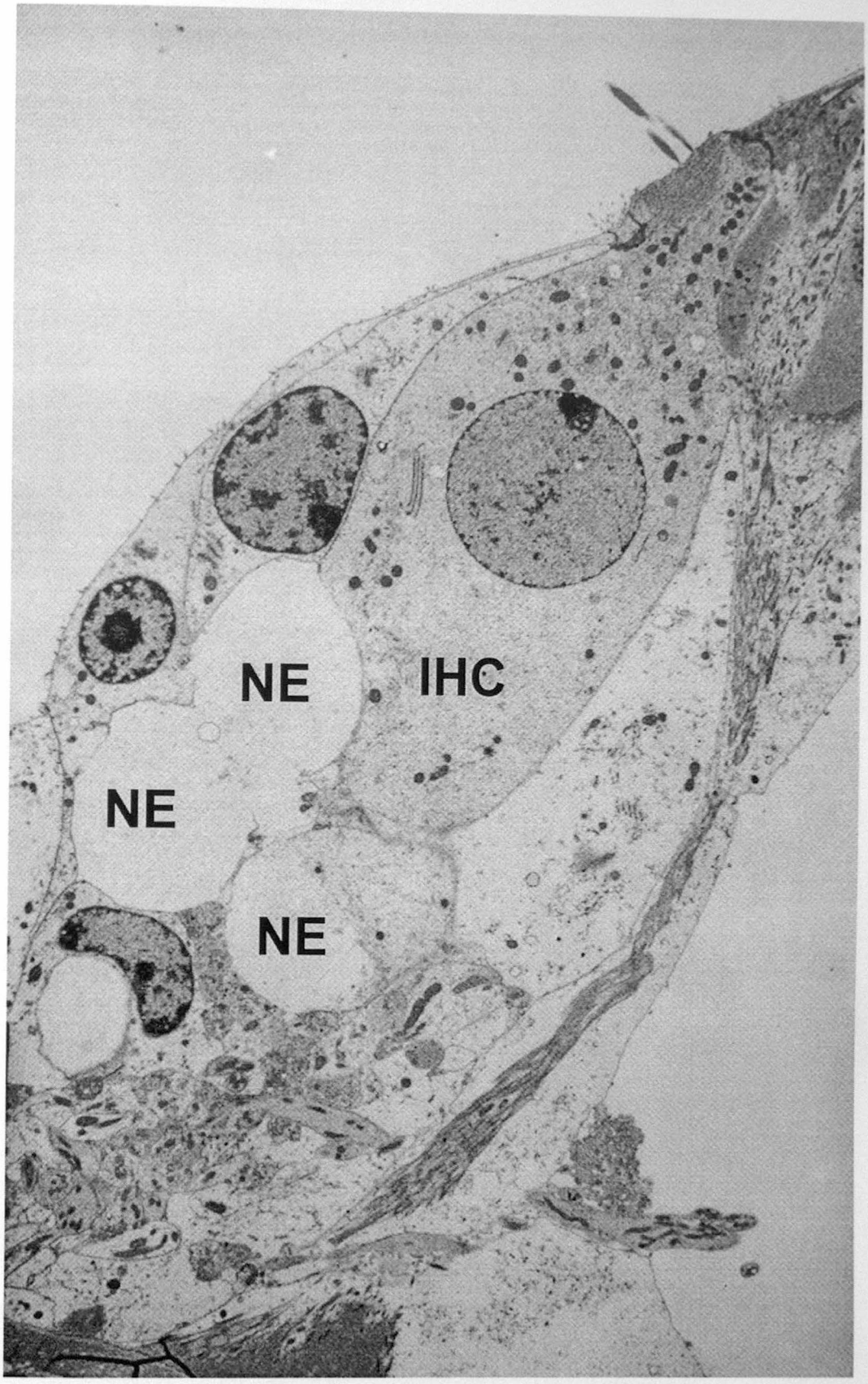


Plate 10iv. Inner hair cell of the left cochlea from animal 10 treated with 10mM quisqualate. Striking swelling to the nerve endings (NEs) at the base of the inner hair cell (IHC). Magnification x 4100

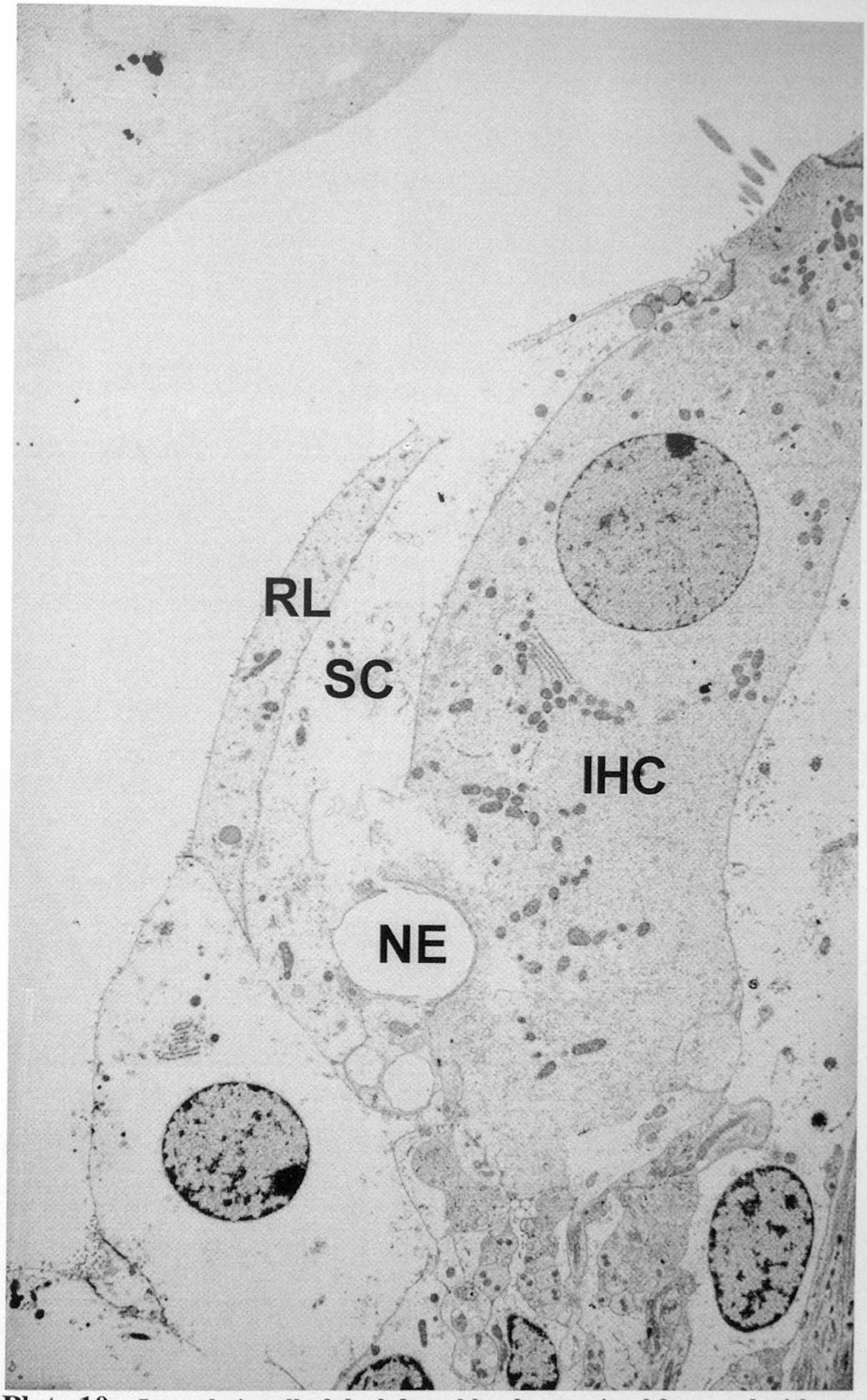


Plate 10v. Inner hair cell of the left cochlea from animal 9 treated with 10mM quisqualate. Swelling of the nerve endings (NEs) at the base of the inner hair cell (IHC) and associated damage to the IHC, supporting cell (SC) and reticular lamina (RL). Magnification x 4100

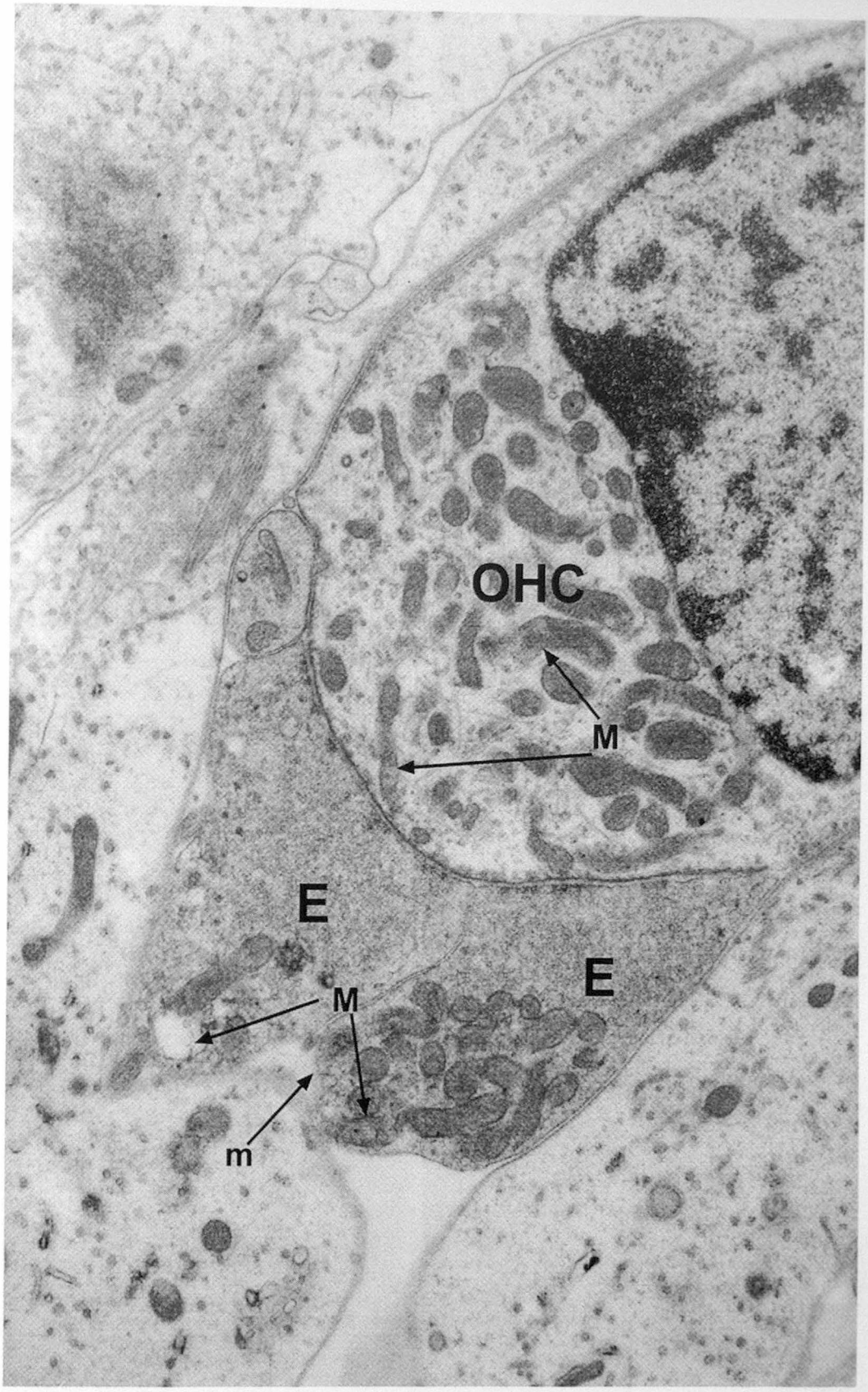


Plate 10vi. A row 3 outer hair cell of the left cochlea from animal 9 treated with 10mM quisqualate. Efferent nerve endings (Es) at the base of the outer hair cell appear to be more dense than in control cochleae, and there is some damage to the mitochondria (M) and membrane (m). Magnification x 22000

- To my knowledge there are no reports of morphological damage produced by quisqualate in cochleae *in vivo*. There have been reports from *in vitro* studies of the effect of quisqualate on cultures of spiral ganglion neurons of the cochlea. Lefebvre, Weber, Leprince *et al.* (1991) reported no neuronal loss within 24h; but their results were not conclusive. Experiments using 50 and 100μM AMPA produced afferent swelling in cochleae with no accompanying damage to the hair cells themselves at both concentrations. At 100μM concentration, AMPA eliminated the CAP, whereas 40 and 80 μM concentrations produced approximately 30 and 50 dB losses (Puel, Pujol, Ladrech *et al.* 1991). Hence, 40-80 μM AMPA produced similar CAP losses to that seen here with 10mM quisqualate and produced a similar extent of morphological damage. Therefore although the two agonists cannot be directly compared, the data appear to be consistent.

10.2 Effect Of Pre-treatment Of Cochleae With Nitric Oxide Synthase (NOS) Inhibitors On The Degree Of Excitotoxicity Produced By Quisqualate And Kainate.

10.2.1 Pre-treatment with Nitro-L-arginine methyl ester (L-NAME)

50mM L-NAME was perfused into cochleae prior to perfusion of kainate and quisqualate in order to determine whether inhibition of NOS protects cochleae against excitotoxicity produced by these non-NMDA agonists. L-NAME was used as hitherto it has been the most commonly used research NOS inhibitor; its effects have been established in many tissues including the cochlea. Previously round window application of 37 and 370 mM L-NAME had been shown to produce effects which are associated with NOS inhibition (e.g. increase in cochlear blood flow) without any losses to the auditory thresholds (Brechtelsbauer, Nuttal and Miller 1994). Since this method depends upon diffusion across the round window membrane, a concentration of 50mM (which is within the 37-371mM range, but towards the lower end) was used to pre-treat cochleae.

10.2.1.1 L-NAME control

50 mM L-NAME was perfused into cochleae of 8 animals (**Table 10.2.1.1**; **Fig. 10.2.1.1**) in order to determine whether L-NAME itself produced any affects on cochlear potentials. Positive CAP losses were displayed by animals 12, 13, 14, 15, 16 and 17, but not by animals 11 and 18. There were no positive CM losses.

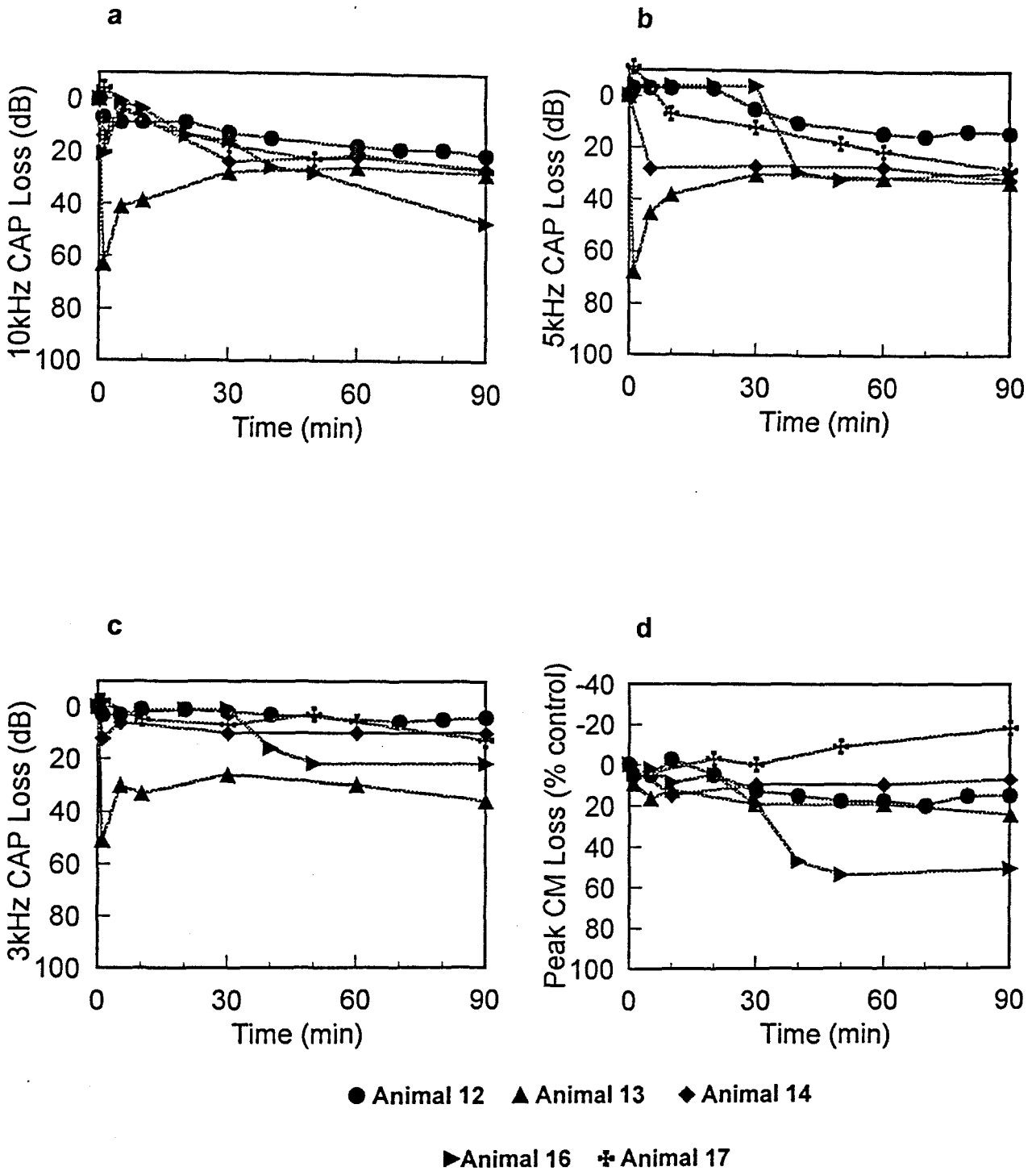


Figure 10.2.1.1. Losses in the CAP and CM in five of the eight animals treated with 50mM L-NAME (data is only presented from animals which had positive losses at 90 min) .

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 10.2.1.1 : Electrophysiological losses evoked by perfusion with 50mM L-NAME in eight animals. Positive losses are bold.

Time post perfusion (min)	Animal 11				Animal 12				Animal 13			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	17	9	39	0.14	18	7	27	0.14	7	-10	22	0.13
	Loss in CAPs (dB) or CM (% control)											
1	19	18	14	-3	7	-3	3	5	**63**	**68**	**51**	9
5	14	7	4	0	9	-3	3	5	**41**	**45**	**30**	16
10	3	3	4	0	9	-3	1	-3	**39**	**38**	**53**	12
30	5	4	2	15	13	5	2	12	**28**	**30**	**26**	19
60	9	2	2	20	18	14	-	17	**26**	**31**	**30**	19
90	11	5	3	20	**21**	14	4	15	**28**	**33**	**36**	24

Time post perfusion (min)	Animal 14				Animal 15				Animal 16			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	24	5	37	0.14	15	-10	58	0.09	9	-6	28	0.10
	Loss in CAPs (dB) or CM (% control)											
1	14	-	12	-	**40**	**62**	13	-	21	-4	-2	5
5	4	**28**	6	-	-	-	-	-	1	-4	2	2
10	-	-	-	15	**46**	**62**	3	-20	4	-4	2	9
30	**24**	**27**	10	10	19	8	-3	16	16	-4	1	19
60	**22**	**27**	10	10	18	8	7	-36	-	-	-	-
90	**26**	**32**	10	7	17	5	-6	-20	**47**	**29**	**22**	**51**

Time post perfusion (min)	Animal 17				Animal 18			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	36	12	32	0.12	40	43	-	0.19
	Loss in CAPs (dB) or CM (% control)							
1	-4	-11	-2	6	27	7	-	-6
5	-	-	-	-	0	5	-	-2
10	9	7	5	-	-7	-2	-	-2
30	18	12	7	0	-10	-5	-	6
60	**21**	**21**	6	-	-8	-5	-	11
90	**27**	**28**	13	-18	-7	-3	-	18

Conclusions

L-NAME produced some ototoxicity in the guinea pig cochlea. However the results were not consistent between animals. Therefore, in all subsequent pre-treatment experiments using L-NAME, perfusions of quisqualate and kainate were not carried out until CAPs had reached a steady level (as judged by more than 2 recordings within 5dB of each other). This method was adopted since it allowed more accurate judgement of the significance of losses produced by kainate and quisqualate. The approach is justified since the *in vivo* effects of L-NAME in the brain have been reported to remain maximal up to 6 h post administration if introduced directly into the CNS (Salter, Duffy, Garthwaite, and Strijbos 1995). Therefore inhibition of NOS should be effective throughout the experiments described hereafter.

10.2.1.2 100μM Kainate following L-NAME Pre-treatment

100μM kainate was perfused into cochleae of animals 15, 19 and 20 which had been pre-treated with L-NAME (**Table 10.2.1.2; Fig. 10.2.1.2**). Data from animal 20 are included in Table 10.2.1.2 and Fig. 10.2.1.2, but are excluded from statistical and summary information as the pre-perfusion thresholds (post L-NAME) were below the defined range for initial auditory thresholds. Positive CAP losses were displayed by animals 19 and 20, but not by animal 15. CAP losses in animal 20 were transient. At 30 min, 10kHz CAP losses were 2, 33 and 10 dB in animals 15, 19 and 20 respectively. There were no positive CM losses seen in any animal.

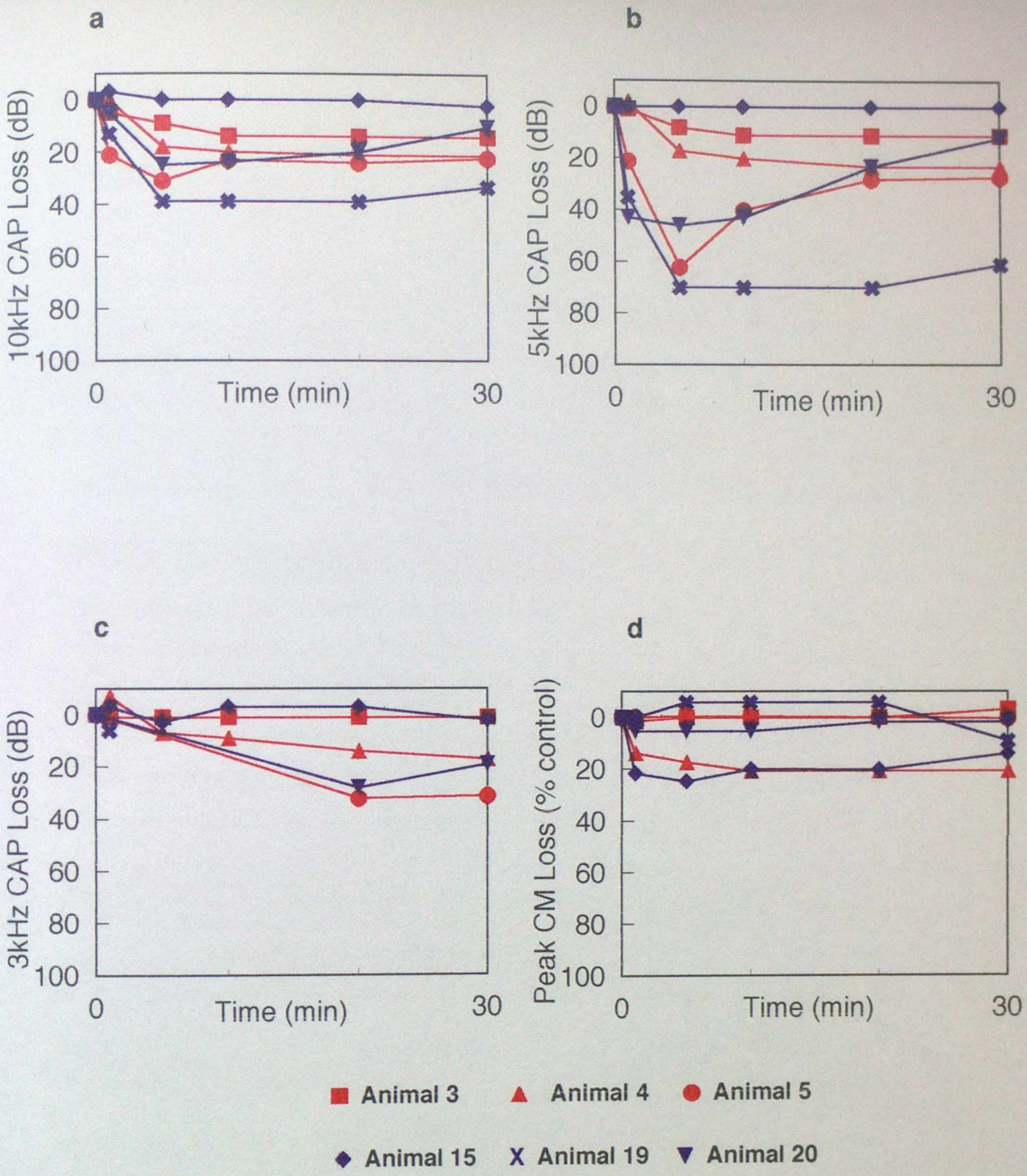


Figure 10.2.1.2. Losses in the CAP and CM in six animals treated with 100μM kainate, three pretreated with 50mM L-NAME (BLUE), and three not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 10.2.1.2 : Electrophysiological losses evoked by perfusion with 100μM kainate in three animals which had been pre-treated with 50mM L-NAME. Positive losses are bold.

Time post perfusion (min)	Animal 15				Animal 19				Animal 20			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0	Pre-perfusion CAPs (dB) and Peak CM (mV) (i.e. post L-NAME)											
(control)	25	-10	43	0.08	32	-5	52	0.10	56	32	52	0.21
	Loss in CAPs (dB) or CM (% control)											
1	-3	0	-3	22	13	**35**	6	0	5	**43**	-	6
5	0	0	3	25	**39**	**70**	-	-6	**25**	**46**	-	6
10	0	0	-3	20	**39**	**70**	-	-6	**24**	**43**	-	6
30	2	0	2	14	**33**	**61**	-	9	10	12	19	2

10.2.1.3. 10mM Kainate following L-NAME Pre-treatment

Since it appeared that L-NAME may have afforded some protection on cochleae from 100μM kainate, its ability to protect cochleae from the action of 10mM kainate was investigated. 10mM kainate was perfused into cochleae of animals 21 and 22 which had be pre-treated with L-NAME **(Table 10.2.1.3; Fig. 10.2.1.3)** and produced large CAP losses at all frequencies in all animals. At 30 min, 10kHz CAP losses were 71, and 44 dB respectively. Positive CM losses of 84% were displayed by animal 21 but not by animal 22.

Table 10.2.1.3 : Electrophysiological losses evoked by perfusion with 10mM kainate in two animals which had been pre-treated with 50mM L-NAME. Positive losses are bold.

Time post perfusion (min)	Animal 21				Animal 22			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0	Pre-perfusion CAPs (dB) and Peak CM (mV) (i.e. post L-NAME)							
(control)	19	12	36	0.13	25	22	43	0.12
	Loss in CAPs (dB) or CM (% control)							
1	**59**	**56**	**52**	**78**	**44**	**52**	**62**	12
5	**61**	**56**	**64**	**73**	**45**	**55**	**60**	9
10	**58**	**54**	**69**	**62**	**45**	**56**	**62**	12
30	**71**	**78**	**69**	**84**	**44**	**52**	**61**	18

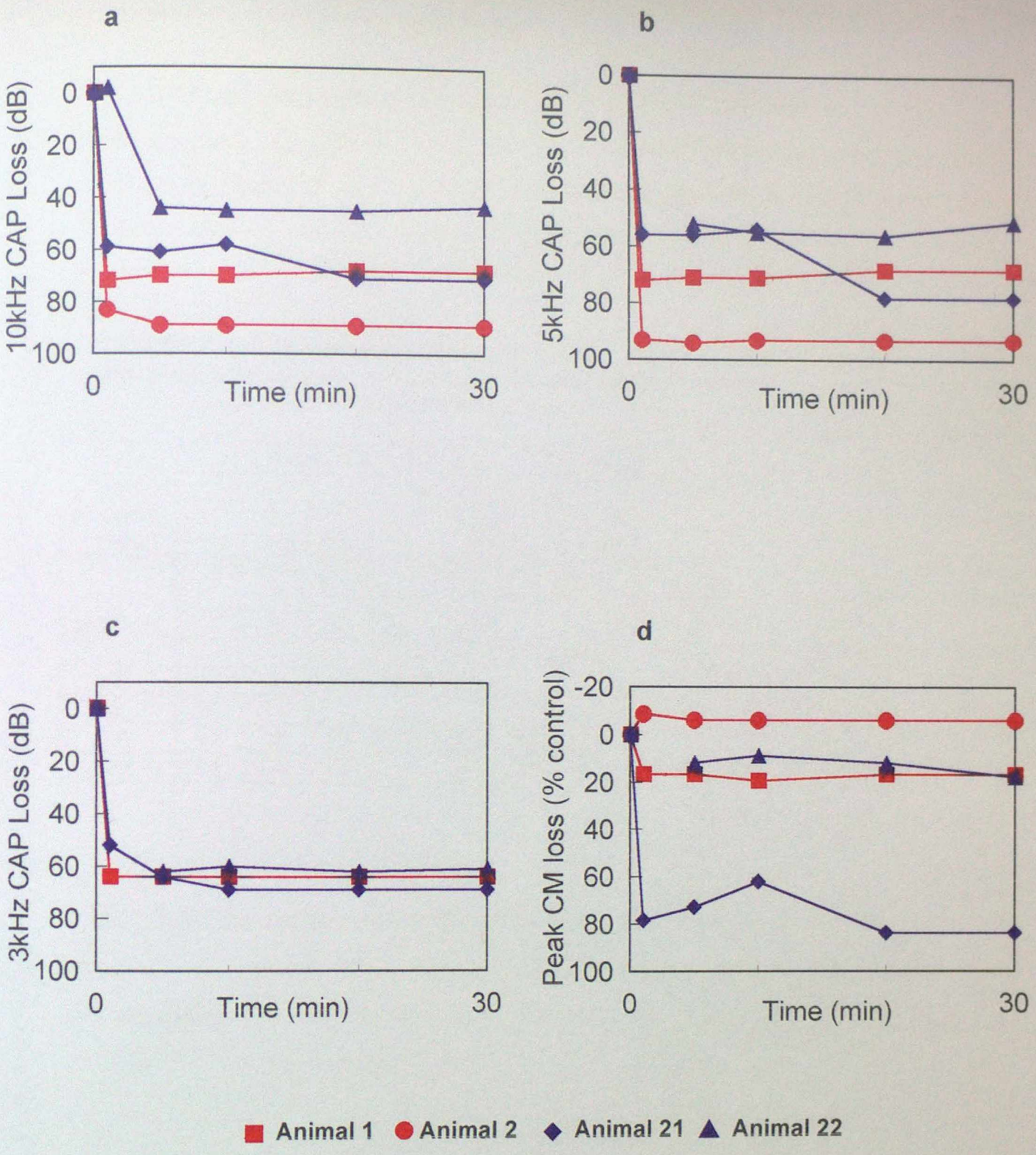


Figure 10.2.1.3. Losses in the CAP and CM in four animals treated with 10mM kainate, two pretreated with 50mM L-NAME (BLUE), and two not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

10.2.1.4 10mM Quisqualate following L-NAME Pre-treatment

10mM quisqualate was perfused into cochleae of animals 23 and 24 which had been pre-treated with L-NAME (**Table 10.2.1.4; Fig. 10.2.1.4**) and produced positive CAP losses in both animals. At 30 min, animal 23 lost 64dB at 10 and 5kHz, and animal 24 lost 65 dB at 5kHz. There were no positive CM losses.

Table 10.2.1.4 : Electrophysiological losses evoked by perfusion with 10mM quisqualate in two animals which had been pre-treated with 50mM L-NAME. Positive losses are bold.

Time post perfusion (min)	Animal 23				Animal 24			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post L-NAME)							
	16	9	35	0.10	23	25	48	0.22
	Loss in CAPs (dB) or CM (% control)							
1	**14**	-2	0	-16	**52**	**65**	-	-17
5	**29**	11	8	-21	**50**	**65**	-	-13
10	**67**	**36**	18	-14	**33**	**65**	-	-13
30	**64**	**64**	18	-14	17	**65**	-	-7

10.2.1.5 Pre-treatment with L-NAME :Summary and Conclusions

30 min CAP and CM losses produced by perfusion of quisqualate and kainate into cochleae with and without prior L-NAME pre-treatment are summarised in **Fig. 10.2.1.5** and **Table 10.2.1.5.**

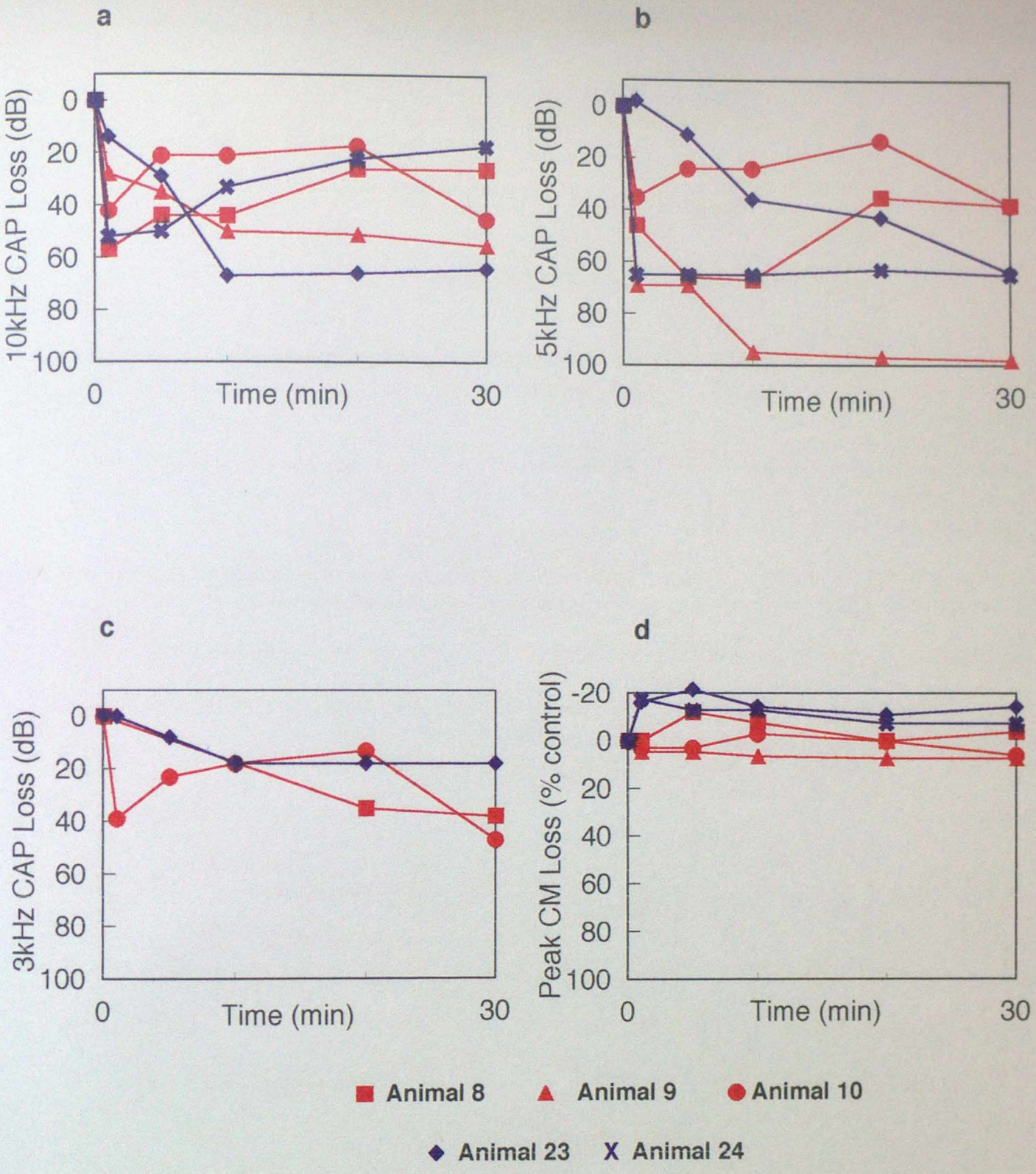


Figure 10.2.1.4. Losses in the CAP and CM in five animals treated with 10mM quisqualate, two pretreated with 50mM L-NAME (BLUE), and three not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

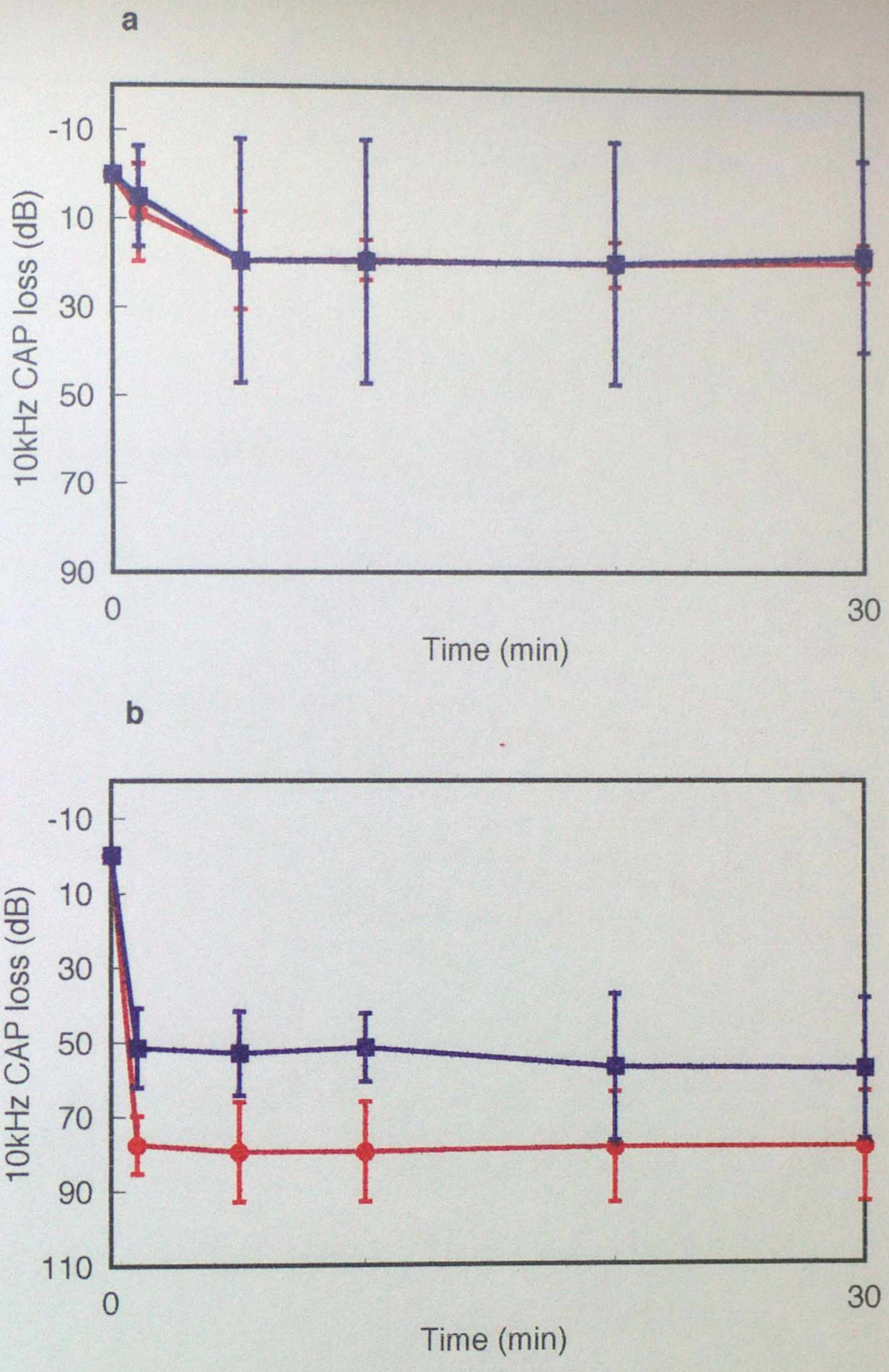


Figure 10.2.1.5. Average (± S.D) 10kHz CAP losses produced by perfusion of kainate and quisqualate with (■) and without(●) pretreament with 50mM L-NAME.

a) 100μM kainate b) 10mM kainate c) 10mM quisqualate

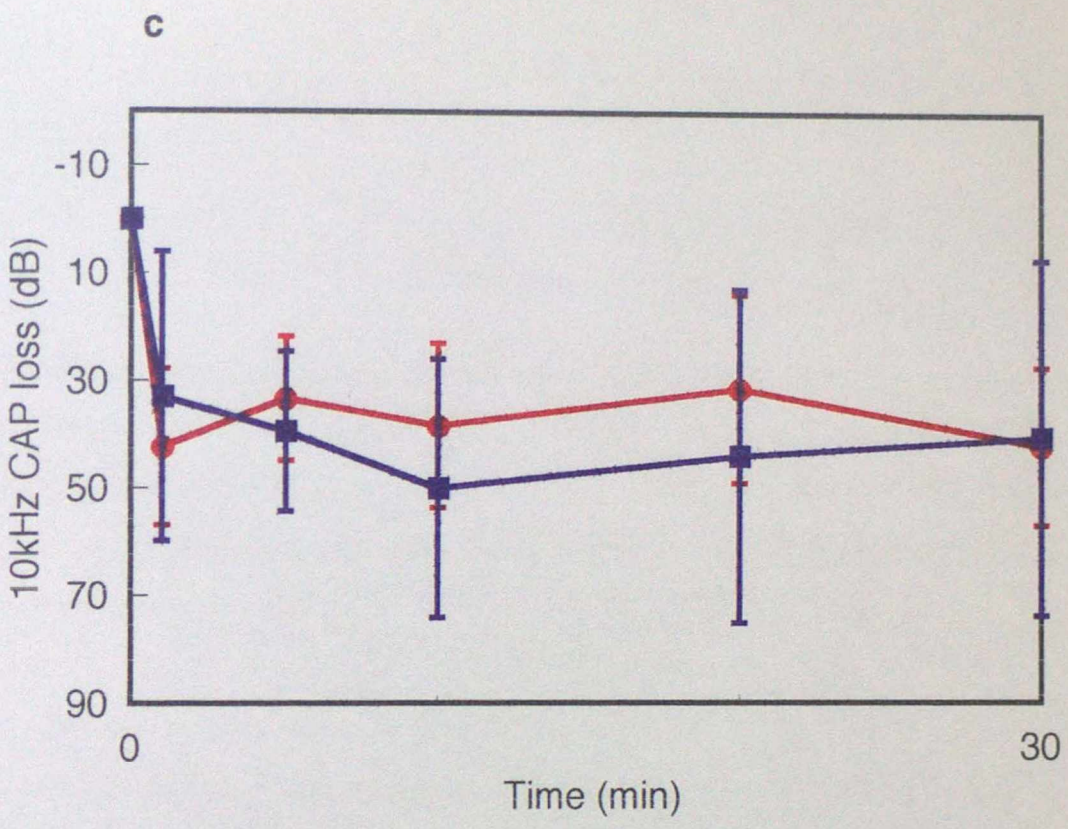


Figure 10.2.1.5. Continued. **Average (± S.D) 10kHz CAP losses produced by perfusion of kainate and quisqualate with (■) and without(●) pretreament with 50mM L-NAME.**

a) 100μM kainate b) 10mM kainate c) 10mM quisqualate

Table 10.2.1.5 : Average 30 min losses in CAPs and CMs produced by perfusion with 10mM and 100μM kainate (KA) and 10mM quisqualate (QA) with or without prior perfusion of L-NAME (+/- respectively). Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 30 min post perfusion			
	10 kHz CAP	5 kHz CAP	3kHz CAP	CM
10mMKA - L-NAME (n=2)	**78 ± 15**	**80 ± 18**	**64**	5 ± 16
10mMKA + L-NAME (n=2)	**58 ± 19**	**65 ± 18**	**65 ± 6**	**51 ± 47**
100μM KA - L-NAME (n=3)	19 ± 4	**20 ± 8**	16 ± 5	6 ± 13
100μM KA + L-NAME (n=2)	18 ± 22	**30 ± 43**	2	12 ± 3
10mM QA - L-NAME (n=3)	**42 ± 15**	**58 ± 35**	**42 ± 6**	3 ± 6
10mM QA + L-NAME (n=2)	**40 ± 33**	**64 ± 1**	18	-11 ± 5

Conclusions

- There was evidence of excitotoxicity produced by 10mM kainate and 10mM quisqualate despite pre-treatment of cochleae with L-NAME. There was some evidence of excitotoxicity produced by 100μM kainate despite pre-treatment with L-NAME, however the results were not consistent between animals.
- Statistical analysis of the 10kHz CAP results indicates that were no significant differences between the data from pre-treated and non-pre-treated cochleae perfused with 10mM kainate, 100μM kainate and 10mM quisqualate with respect to treatment alone ($p= 0.2$, 0.95 and 0.6 respectively), or the change in the CAP with time ($p= 0.7$, 0.98 and 0.1 respectively).
- Therefore L-NAME provided no protection on cochleae from the effects of kainate and quisqualate at the concentrations used.

10.2.2 Pre-treatment with L-Methyl arginine (L-MA)

Since L-MA had previously been reported to protect cochleae from several ototoxic insults (Amaee *et al.* 1997), the hypothesis that NOS inhibition may protect cochleae against the action of quisqualate and kainate was further examined by pre-treatment of cochleae with L-MA. L-MA is an inhibitor of NOS but is less potent at inhibiting nNOS than L-NAME (Korytko and Boje 1996). 50mM concentration was used because this was the concentration at which the compound

afforded protection to cochleae against the action of NMDA and pneumolysin (Amaee *et al.* 1997).

10.2.2.1 L-MA control

In order to assess whether L-MA itself produced similar ototoxicity to L-NAME, 50 mM L-MA was tested for ototoxicity in 9 animals (**Table 10.2.2.1**; **Fig. 10.2.2.1**). Data from animals 25 and 26 are included in Table 10.2.2.1 and Fig. 10.2.2.1, but have been excluded from statistical and summary information as the animals displayed initial auditory thresholds outside the defined range. Positive CAP losses were displayed by animals 25, 27, 28, 30, 31, 32 and 33, but not by animals 26 and 29. The CAP losses were transient in animals 25, 30 and 32 with no positive CAP losses at 60 min. Positive CM loss was displayed by animal 25 only, although animal 27 displayed positive increases in the size of the CM.

Conclusions

- L-MA produced some excitotoxicity in the guinea pig cochlea. However the results were not consistent between animals. Therefore, in all pre-treatment experiments using L-MA, perfusions of quisqualate and kainate were not carried out until CAPs had reached a steady level (as judged by more than 2 recordings within 5dB of each other). This method was adopted since it allowed more accurate judgement of the significance of losses produced by kainate and quisqualate and was justifiable since the *in vivo* effects of L-MA have been reported to be irreversible (Korytko and Boje 1996). Therefore it is highly likely that inhibition of NOS would have been effective throughout the experiments described here.

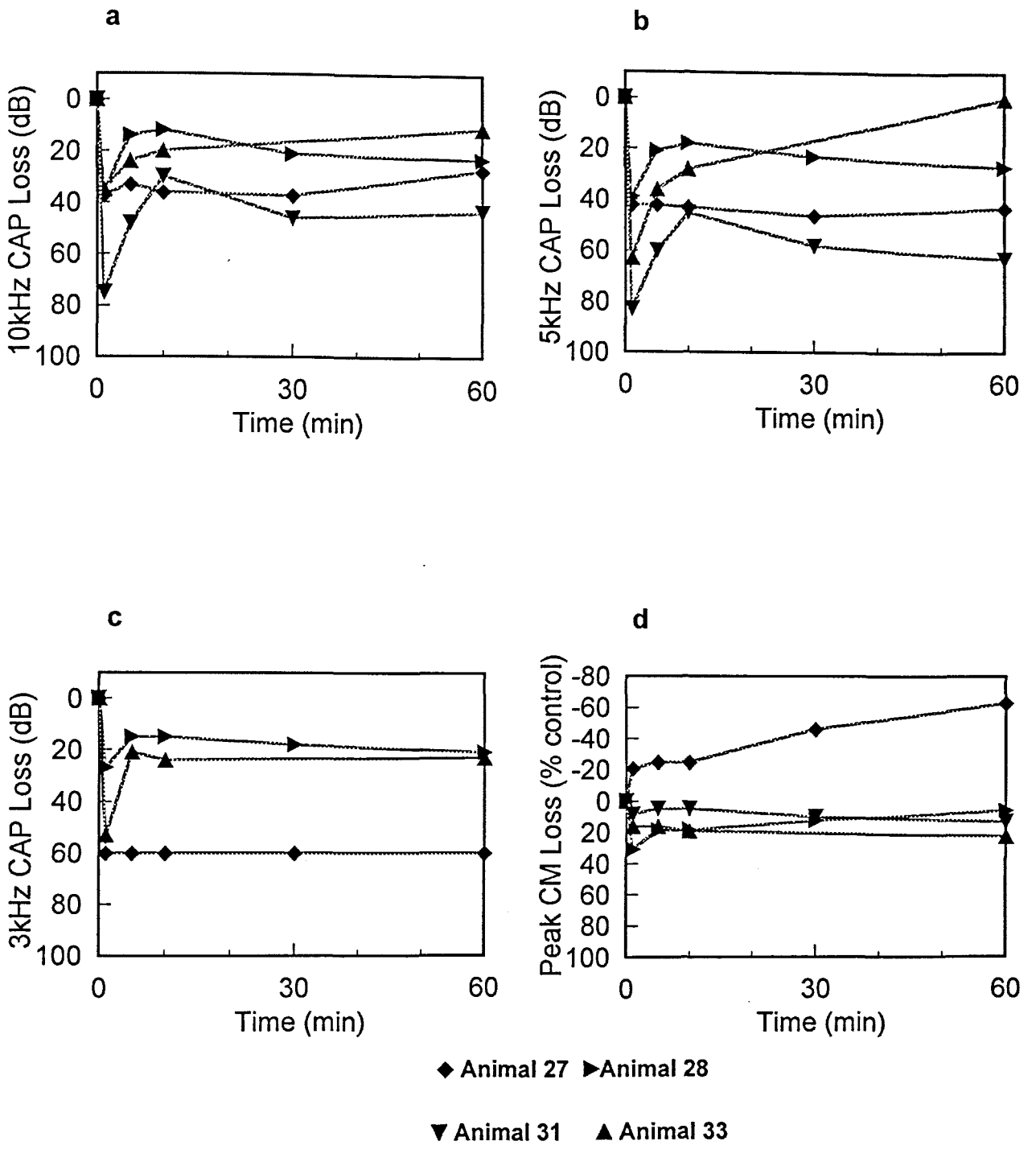


Figure 10.2.2.1. Losses in the CAP and CM in four out of nine animals treated with 50mM L-MA (data is only presented from animals which displayed positive losses at 60 min).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 10.2.2.1 : Electrophysiological losses evoked by perfusion with 50mM L-MA in nine animals. Positive losses are bold.

Time post perfusion (min)	Animal 25				Animal 26				Animal 27			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	56	48	53	0.18	53	40	51	0.14	30	22	45	0.08
	Loss in CAPs (dB) or CM (% control)											
1	**22**	**21**	**37**	35	12	11	-	-5	**37**	**42**	**60**	-21
5	19	**21**	**35**	30	12	13	-	-8	**33**	**42**	**60**	-25
10	19	**20**	**37**	**41**	7	8	14	-10	**36**	**43**	**60**	-25
30	10	10	12	33	2	1	4	-5	**37**	**46**	**60**	-46
60	11	9	11	37	-2	-2	-2	-5	**27**	**43**	**60**	-63
90	-	-	-	-	-	-	-	-	**27**	**45**	**60**	-67

Time post perfusion (min)	Animal 28				Animal 29				Animal 30			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	29	19	43	0.06	37	22	45	0.05	25	-10	45	0.04
	Loss in CAPs (dB) or CM (% control)											
1	**36**	**39**	**27**	31	13	8	10	7	**30**	**52**	7	-18
5	14	**21**	15	19	-2	-2	3	7	10	**30**	0	-18
10	12	18	15	19	-2	1	3	14	8	18	-2	-18
30	**21**	**23**	18	12	1	-2	2	7	5	7	-2	-18
60	**23**	**27**	**21**	6	-	-	-	-	5	10	0	0
90	-	-	-	-	-	-	-	-	-	-	-	-

Time post perfusion (min)	Animal 31				Animal 32				Animal 33			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	-5	-10	5	0.21	10	-10	5	0.09	20	-10	15	0.11
	Loss in CAPs (dB) or CM (% control)											
1	**75**	**83**	-	8	**47**	**60**	**65**	15	**35**	**63**	**53**	16
5	**48**	**60**	-	5	**36**	**53**	**44**	15	**24**	**36**	**21**	16
10	**30**	**45**	-	5	**27**	**46**	**40**	0	**20**	**28**	**24**	19
30	**46**	**58**	-	10	15	**22**	**20**	7	-	-	-	-
60	**43**	**63**	-	13	15	6	6	0	11	0	**23**	23
90	-	-	-	-	-	-	-	-	-	-	-	-

10.2.2.2 100μM Kainate following L-MA Pre-treatment

100μM kainate was perfused into cochleae of animals 25, 26, 27 and 28 which had been pre-treated with L-MA (**Table 10.2.2.2; Fig. 10.2.2.2**). All animals tested displayed pre-perfusion (post L-MA) CAP thresholds outside the defined range for initial auditory thresholds, therefore data from this treatment group are interpreted with caution and will be bracketed when presented in statistical and summary information. Positive CAP losses were displayed by animal 27, but not by animals 25, 26 and 28. At 30 min, 10kHz CAP losses were 12, 15, 38 and 0 dB in animals 25, 26, 27 and 28 respectively. There were no positive CM losses seen in any animal.

Table 10.2.2.2 : Electrophysiological losses evoked by perfusion with 100μM kainate in two animals which had been pre-treated with 50mM L-MA. Positive losses are bold.

Time post perfusion (min)	Animal 25				Animal 26			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post L-MA)							
	67	57	64	0.10	51	38	49	0.15
	Loss in CAPs (dB) or CM (% control)							
1	2	4	6	33	0	-2	0	-3
5	-	8	9	30	16	11	-	-2
10	13	9	9	23	16	13	-	-3
30	12	7	13	23	15	12	-	0

Time post perfusion (min)	Animal 27				Animal 28			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post L-MA)							
	57	67	-	0.14	52	46	64	0.06
	Loss in CAPs (dB) or CM (% control)							
1	11	-1	-	9	-2	-1	-1	-14
5	**38**	**23**	-	15	0	-1	-2	-14
10	**33**	**22**	-	17	0	-1	-2	-14
30	**38**	**23**	-	17	0	4	1	-2

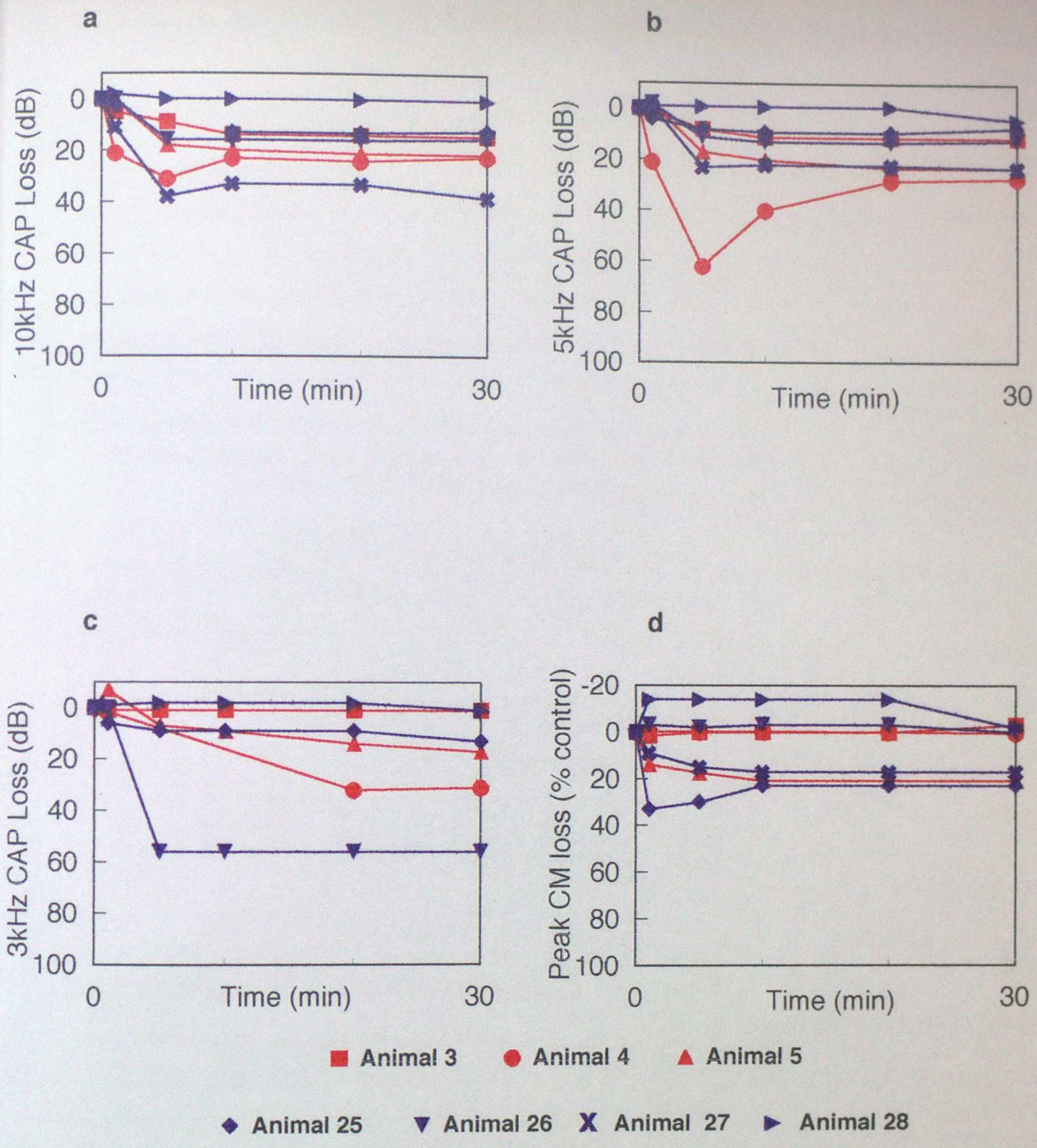


Figure 10.2.2.2. Losses in the CAP and CM in seven animals treated with 100μM kainate, four pretreated with 50mM L-MA (BLUE), and three not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

10.2.2.3 10mM Kainate following L-MA Pre-treatment

Since it appeared that L-MA may have afforded some protection on cochleae from 100μM kainate, experiments were repeated with 10mM kainate. 10mM kainate was perfused into cochleae of animals 29 and 30 which had been pre-treated with 50mM L-MA **(Table 10.2.2.3; Fig. 10.2.3)** and eliminated all CAPs in all animals. At 30 min, 10kHz CAP losses were 57 and 65 dB in animals 29 and 30 respectively. Positive CM losses were not seen in either animal.

Table 10.2.2.3 : Electrophysiological losses evoked by perfusion with 10mM kainate in two animals which had been pre-treated with 50mM L-MA. Positive losses are bold.

Time post perfusion (min)	Animal 29				Animal 30			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post L-MA)							
	38	20	47	0.05	30	0	45	0.04
	Loss in CAPs (dB) or CM (% control)							
1	**57**	**70**	**58**	-31	**65**	**90**	**60**	3
5	**57**	**70**	**58**	-34	**65**	**90**	**60**	9
10	**57**	**70**	**58**	-31	**65**	**90**	**60**	16
30	**57**	**70**	**58**	-17	**65**	**90**	**60**	16

10.2.2.4 10mM Quisqualate following L-MA Pre-treatment

10mM quisqualate was perfused into cochleae of animals 31 and 32 which had been pre-treated with L-MA **(Table 10.2.2.4; Fig. 10.2.2.4)**. Data from animal 32 are included in Table 10.2.2.4 and Fig. 10.2.2.4, but have been excluded from statistical and summary information as the animal displayed pre-perfusion (i.e. post L-MA) auditory thresholds above the defined level. Positive CAP losses were displayed in both animals. At 30 min, 10kHz CAP losses were 24 and 27dB in animal 31 and 32 respectively. There were no positive CM losses.

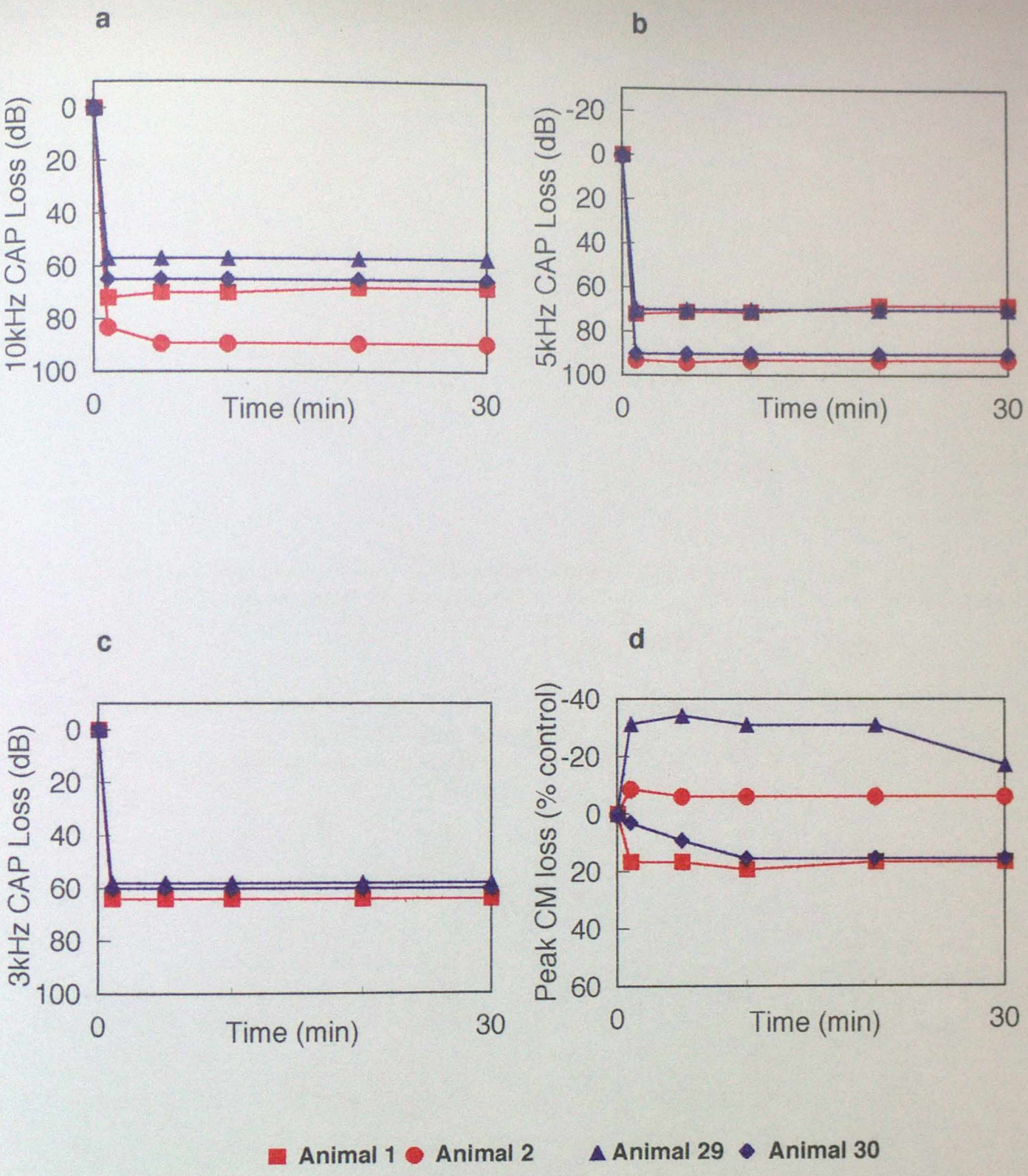


Figure 10.2.2.3. Losses in the CAP and CM in four animals perfused with 10mM kainate, two pretreated with 50mM L-MA (BLUE), and two not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

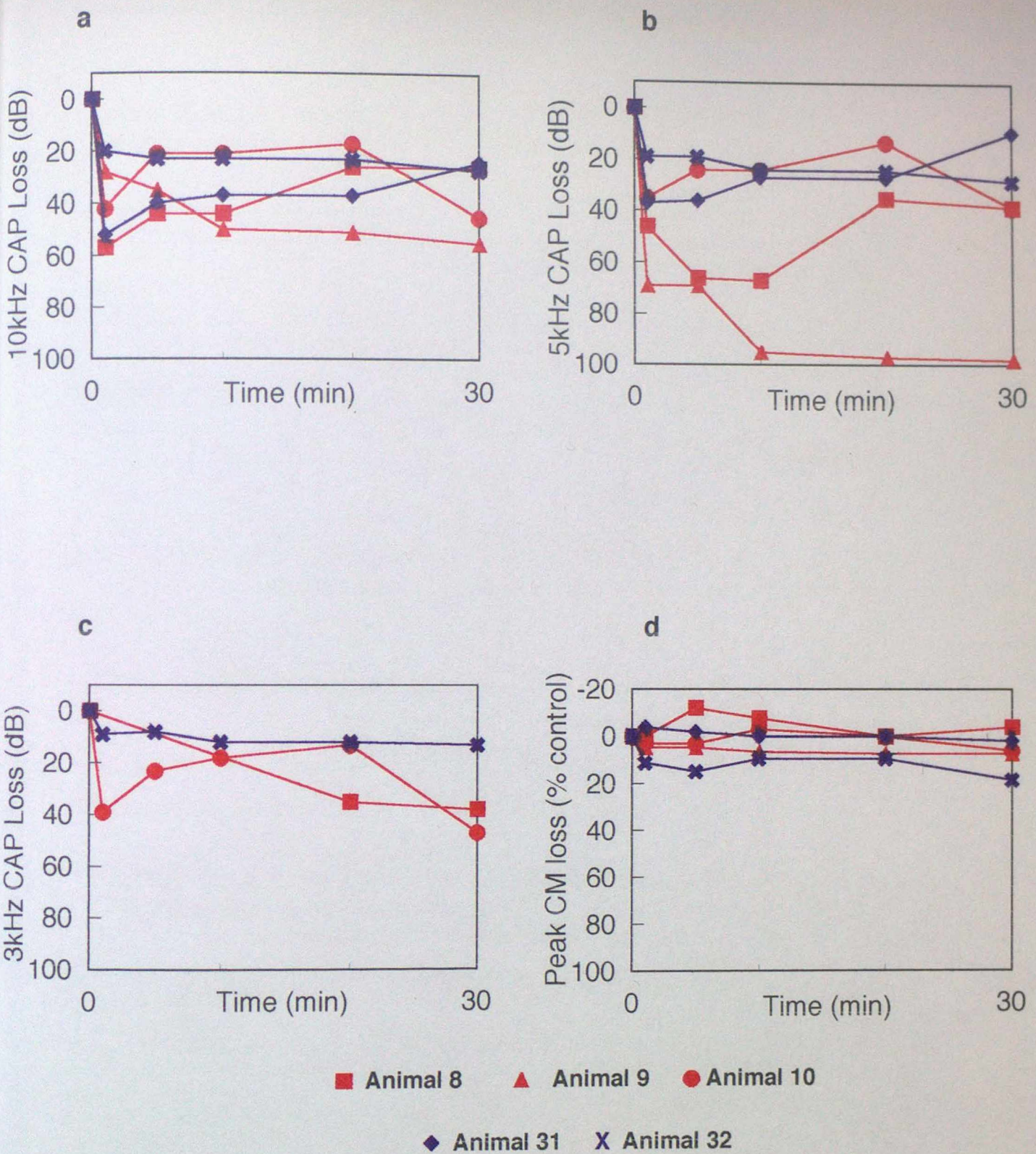


Figure 10.2.2.4. Losses in the CAP and CM in five animals treated with 10mM quisqualate, two pretreated with 50mM L-MA (BLUE), and three not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 10.2.2.4 : Electrophysiological losses evoked by perfusion with 10mM quisqualate in two animals which had been pre-treated with 50mM L-MA. Positive losses are bold.

Time post perfusion (min)	Animal 31				Animal 32			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post L-MA)							
	38	53	-	0.19	25	-4	11	0.09
	Loss in CAPs (dB) or CM (% control)							
1	**52**	**37**	-	-4	**20**	19	9	11
5	**40**	**36**	-	-2	**23**	19	8	15
10	**37**	**27**	-	0	**23**	**24**	12	10
30	**24**	9	-	2	**27**	**28**	13	19

10.2.2.5 Pre-treatment with L-MA :Summary and Conclusions

30 min CAP and CM losses produced by perfusion of kainate and quisqualate in cochleae with and without L-MA pre-treatment are summarised in **Fig. 10.2.2.5** and **Table 10.2.2.5.**

Table 10.2.2.5 : Average 30 min losses in CAPs and CMs produced by perfusion with 10mM and 100μM kainate (KA) and 10mM quisqualate (QA) with or without prior perfusion of L-MA (+/- respectively). Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 30 min post perfusion Bracketed results are from animals with pre-perfusion thresholds above the defined level			
	10 kHz CAP	5 kHz CAP	3kHz CAP	CM
10mMKA - L-MA (n=2)	**78 ± 15**	**80 ± 18**	**64**	5 ± 16
10mMKA+ L- MA (n=2)	**61 ± 6**	**80 ± 14**	**59 ± 1**	-1 ± 23
100μM KA - L- MA (n=3)	19 ± 4	**20 ± 8**	16 ± 5	6 ± 13
100μM KA + L- MA (n=4)	(16 ± 16)	(12 ± 8)	(7 ± 8)	(10 ± 12)
10mM QA - L- MA (n=3)	**42 ± 15**	**58 ± 35**	**42 ± 6**	3 ± 6
10mM QA + L- MA (n=1)	**27**	28	13	19

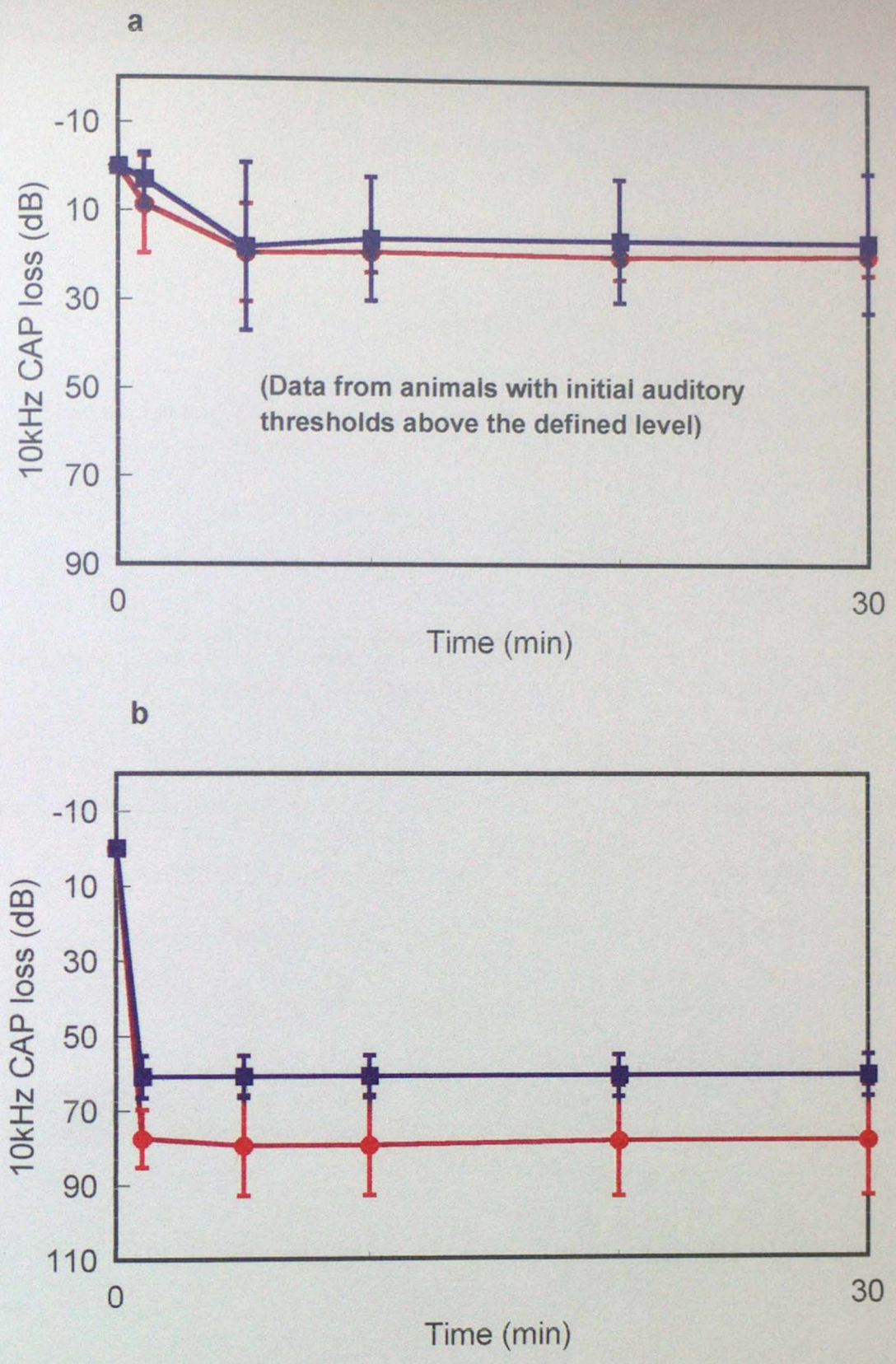


Figure 10.2.2.5. **Average (± S.D) 10kHz CAP losses produced by perfusion of kainate and quisqualate with (■) and without(●) pretreament with 50mM L-MA.**

a) (100μM kainate) b) 10mM kainate c) 10mM quisqualate

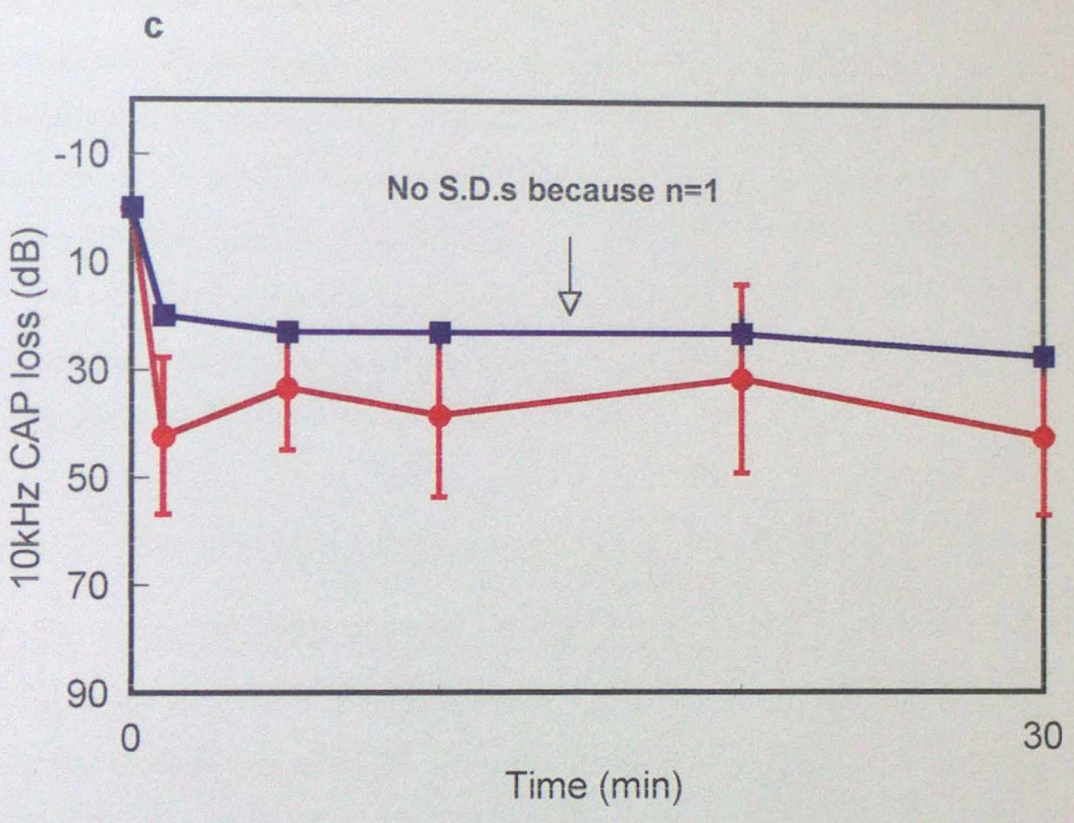


Figure 10.2.2.5. Continued. Average (± S.D) 10kHz CAP losses produced by perfusion of kainate and quisqualate with (■) and without(●) pretreament with 50mM L-MA.

a) (100μM kainate) b) 10mM kainate c) 10mM quisqualate

Conclusions

- There was evidence of excitotoxicity produced by 10mM kainate and quisqualate despite pre-treatment of cochleae with L-MA. There was some evidence of excitotoxicity produced by 100μM kainate despite pre-treatment with L-MA, however the results were not consistent between animals, and all animals presented pre-perfusion thresholds outside the defined range (i.e. all animals had positive existing hearing losses due to L-MA perfusions).
- Statistical analysis of 10kHz CAP losses by ANOVA demonstrated no significant differences between L-MA pre-treated and non pre-treated data from cochleae perfused with either 10mM kainate, 100μM kainate or 10mM quisqualate with respect to treatment alone ($p= 0.2$, 0.8 and 0.5 respectively), or the change in the CAP with time ($p= 0.95$, 0.94 and 0.89 respectively).
- Therefore L-MA provided no protection on cochleae from the effects of kainate and quisqualate at the concentrations used.

10.2.3 Morphological Examinations

10.2.3.1. 10mM Kainate following L-NAME pre-treatment

Cochleae pre-treated with L-NAME and perfused with 10mM kainate revealed widespread damage to the base of the inner hair cell and surrounding structures similar to that seen in non-pre-treated cochleae.

10.2.3.2. 10mM Quisqualate following L-NAME pre-treatment

Cochleae pre-treated with L-NAME and perfused with 10mM quisqualate revealed widespread damage to the afferent nerve terminals at the base of the inner hair cell with some associated damage to surrounding structures, very similar to that seen in non-pre-treated cochleae.

Conclusion

There was no morphological evidence that L-NAME afforded any protection on cochleae from the excitotoxic action of either quisqualate or kainate at the concentrations used.

10.2.4 Effect Of Pre-treatment With NOS Inhibitors On The Degree Of Excitotoxicity Produced By Kainate And Quisqualate : Summary And Conclusions

30 min 10kHz CAP losses produced by perfusion of quisqualate or kainate in cochleae with and without pre-treatment with NOS inhibitors are summarised in **Fig. 10.2.4**.

Conclusions

- At the concentrations used, neither L-NAME nor L-MA pre-treatment afforded any protection on cochleae from the excitotoxic action of either kainate or quisqualate as judged by electrophysiological and morphological criteria.

- The data could be interpreted in two ways.

  (i) Both inhibitors did not inhibit NOS production. However, perfusion of both inhibitors produced some changes in the size of the CAP, which suggests that *per se* they did have some effect on cochlear function. In the case of L-MA it had previously been shown to be active within cochleae at these concentrations in inhibiting the effects of NMDA and pneumolysin (Amaee *et al.* 1997 and Brechtelsbauer *et al.* 1994).

  (ii) Alternatively, the data could mean that production of NO by NOS is not involved in the excitotoxicity produced by kainate and quisqualate at the concentrations used.

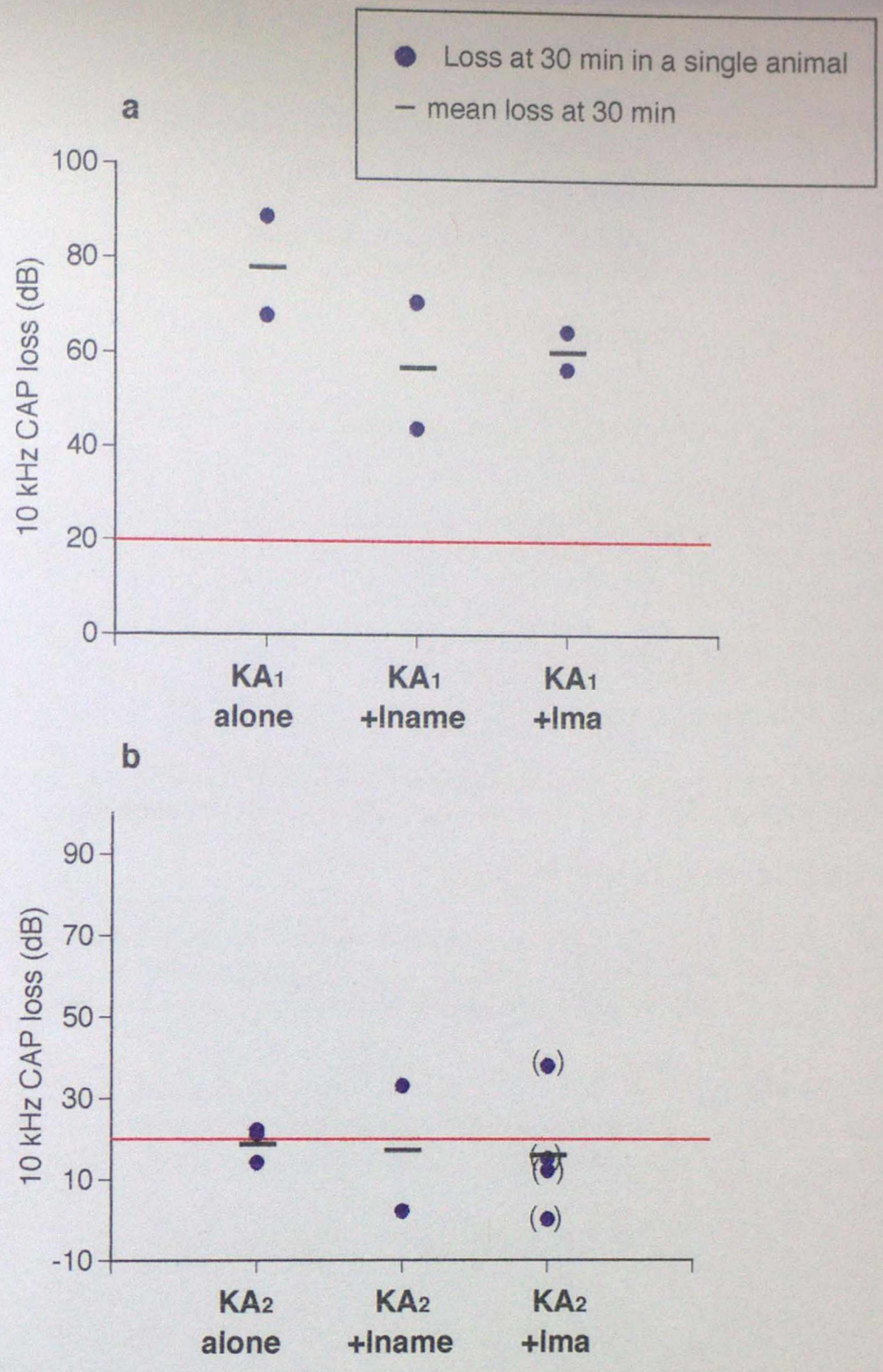


Figure 10.2.4. **30 min 10kHz CAP losses produced by perfusion of kainate and quisqualate with and without pretreament with NOS inhibitors L-NAME/L-MA (lname/lma).**

a) 10mM kainate perfusions b) 100 M kainate perfusions

Bracketed data are from animals with pre-perfusion (post L-MA) thresholds above the defined level.

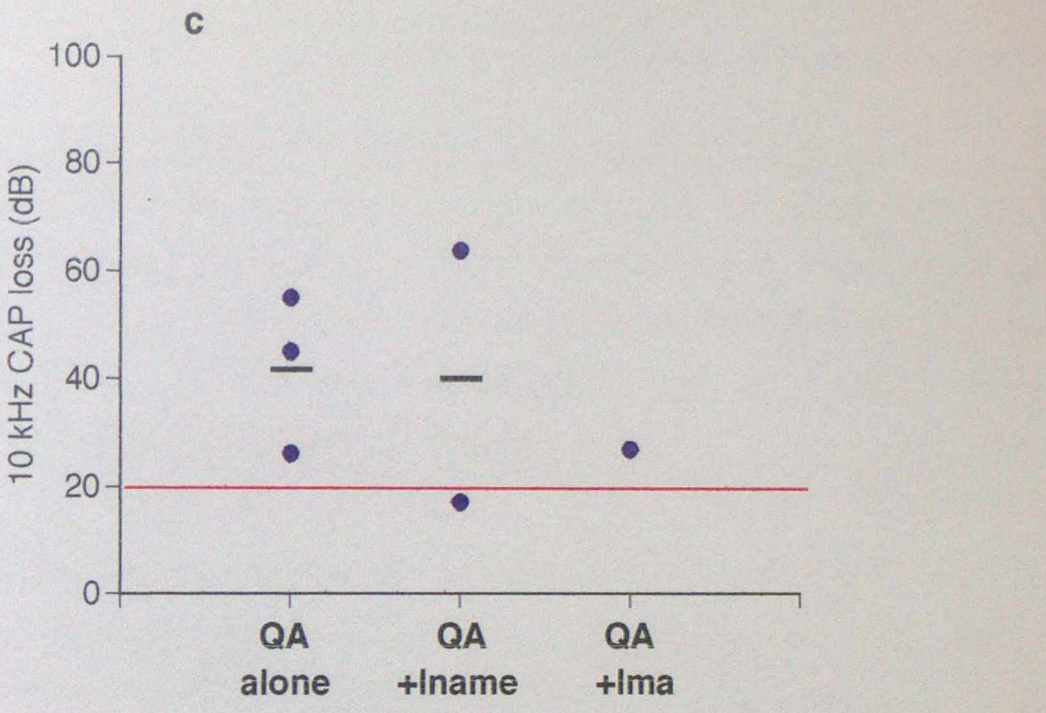


Figure 10.2.4. continued. **30 min 10kHz CAP losses produced by perfusion of kainate and quisqualate with and without pretreament with NOS inhibitors L-NAME/L-MA (lname/lma) .**

c) 10mM quisqualate perfusions

10.3 Effect Of Pre-treatment Of Cochleae With Free Radical Scavengers On The Degree Of Excitotoxicity Produced By Quisqualate And Kainate.

10.3.1 Pre-treatment with Superoxide Dismutase (SOD)

SOD was perfused into cochleae prior to perfusion of kainate and quisqualate in order to determine whether scavenging for superoxide protects cochleae against excitotoxicity produced by these non-NMDA agonists. SOD is a scavenger of the superoxide radical and has been demonstrated to protect successfully cochleae from the ototoxic effects of excessive exposure to superoxide at 0.05 unitsμl^{-1} (Clerici and Yang 1996). It has also been demonstrated to afford protection on cochleae from the excitotoxic action of NMDA at 1560 unitsμl^{-1} (Amaee *et al.* 1997). Therefore SOD was used at a concentration of 0.1 unitsμl^{-1} in this study.

10.3.1.1 SOD control

100 uml^{-1} SOD was perfused into cochleae of animals 34, 35, 36 and 37 (**Table 10.3.1.1**) in order to determine whether SOD itself produced any affects on cochlear potentials. No animal displayed positive losses in CAPs or CMs within 30 min.

Table 10.3.1.1 : Electrophysiological losses evoked by perfusion with SOD in four animals. Positive losses are bold.

Time post perfusion (min)	Animal 34				Animal 35			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	18	13	39	0.19	25	18	46	0.20
	Loss in CAPs (dB) or CM (% control)							
1	-8	-3	-	-8	-5	2	4	3
5	-	-	-1	-	-5	6	4	3
10	0	-3	2	-2	-5	4	-	3
30	2	2	-	-6	-	-	-	-

Time post perfusion (min)	Animal 36				Animal 37			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)							
	15	-10	26	0.14	18	15	39	0.15
	Loss in CAPs (dB) or CM (% control)							
1	18	15	7	-5	2	0	-3	-12
5	8	9	9	0	0	-1	-1	-7
10	10	13	14	5	0	-2	-3	-12
30	10	10	10	2	-	-	-	-

Conclusions

There was no evidence from the electrophysiological data of ototoxicity produced by SOD, therefore kainate and quisqualate were perfused into cochleae of these animals without any pre-existing damage having been caused by perfusion of SOD.

10.3.1.2 100μM Kainate following SOD Pre-treatment

100μM kainate was perfused into cochleae of animals 34 and 35 which had been pre-treated with 100 uml^{-1} SOD (**Table 10.3.1.2; Fig. 10.3.1.2**) and produced positive CAP losses at all frequencies in both animals. At 30 min, 10kHz CAP losses were 53 and 58 dB in animals 34 and 35 respectively. There were no positive CM losses.

Table 10.3.1.2 : Electrophysiological losses evoked by perfusion with 100μM kainate in two animals which had been pre-treated with SOD. Positive losses are bold.

Time post perfusion (min)	Animal 34				Animal 35			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post SOD)							
	20	15	41	0.20	20	22	-	0.19
	Loss in CAPs (dB) or CM (% control)							
1	-2	3	4	-12	**58**	**61**	-	9
5	-2	-	4	-12	**58**	**63**	-	13
10	**65**	**75**	**64**	-12	**59**	**63**	-	16
30	**53**	**75**	**64**	-16	**58**	**59**	-	13

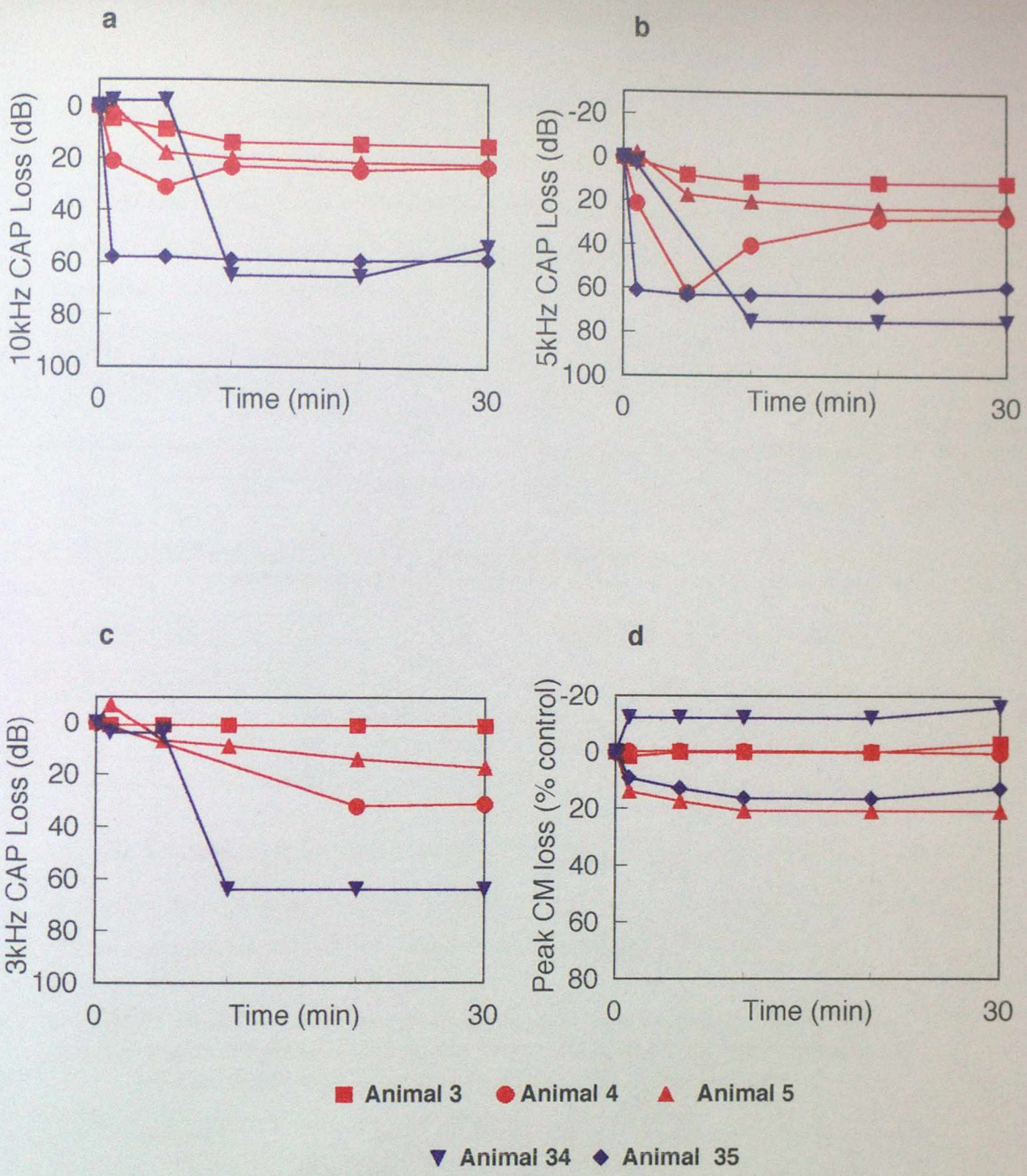


Figure 10.3.1.2. Losses in the CAP and CM in five animals perfused with 100μM kainate, two pretreated with SOD (BLUE), and three not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively.
d) Loss in the CM elicited with a 5kHz stimuli.

10.3.1.3 10mM Quisqualate With SOD Pre-treatment

10mM quisqualate was perfused into cochleae of animals 36 and 37 which had been pre-treated with 100 uml^{-1} SOD (**Table 10.3.1.3; Fig. 10.3.1.3**) and produced positive CAP losses at all frequencies in both animals. At 30 min, 10kHz CAP losses were 53 and 30dB in animals 36 and 37 respectively. There were no positive CM losses.

Table 10.3.1.3 : Electrophysiological losses evoked by perfusion with 10mM quisqualate in two animals which had been pre-treated with SOD. Positive losses are bold.

Time post perfusion (min)	Animal 36				Animal 37			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post SOD)							
	25	0	36	0.14	18	13	36	0.17
	Loss in CAPs (dB) or CM (% control)							
1	**48**	**71**	-	-2	**27**	**20**	-	4
5	**51**	**71**	**69**	0	**27**	**21**	-	1
10	**53**	**73**	**69**	0	**30**	**22**	-	3
30	**53**	**72**	**69**	-2	**30**	**21**	-	1

10.3.1.4 Pre-treatment with SOD :Summary and Conclusions

30 min CAP and CM losses produced by perfusion of kainate and quisqualate in cochleae with and without pre-treatment with SOD are summarised in **Fig. 10.3.1.4** and **Table 10.3.1.4.**

Table 10.3.1.4 : Average 30 min losses in CAPs and CMs produced by perfusion with 100µM kainate (KA) and 10mM quisqualate (QA) with or without prior perfusion of SOD (+/- respectively). Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 30 min post perfusion			
	10 kHz CAP	5 kHz CAP	3kHz CAP	CM
100µM KA - SOD (n=3)	19 ± 4	**20 ± 8**	16 ± 5	6 ± 13
100µM KA + SOD (n=2)	**56 ± 4**	**67 ± 11**	**64**	-2 ± 21
10mM QA - SOD (n=3)	**42 ± 15**	**58 ± 35**	**42 ± 6**	3 ± 6
10mM QA + SOD (n=2)	**42 ± 16**	**39 ± 13**	**26 ± 21**	21 ± 50

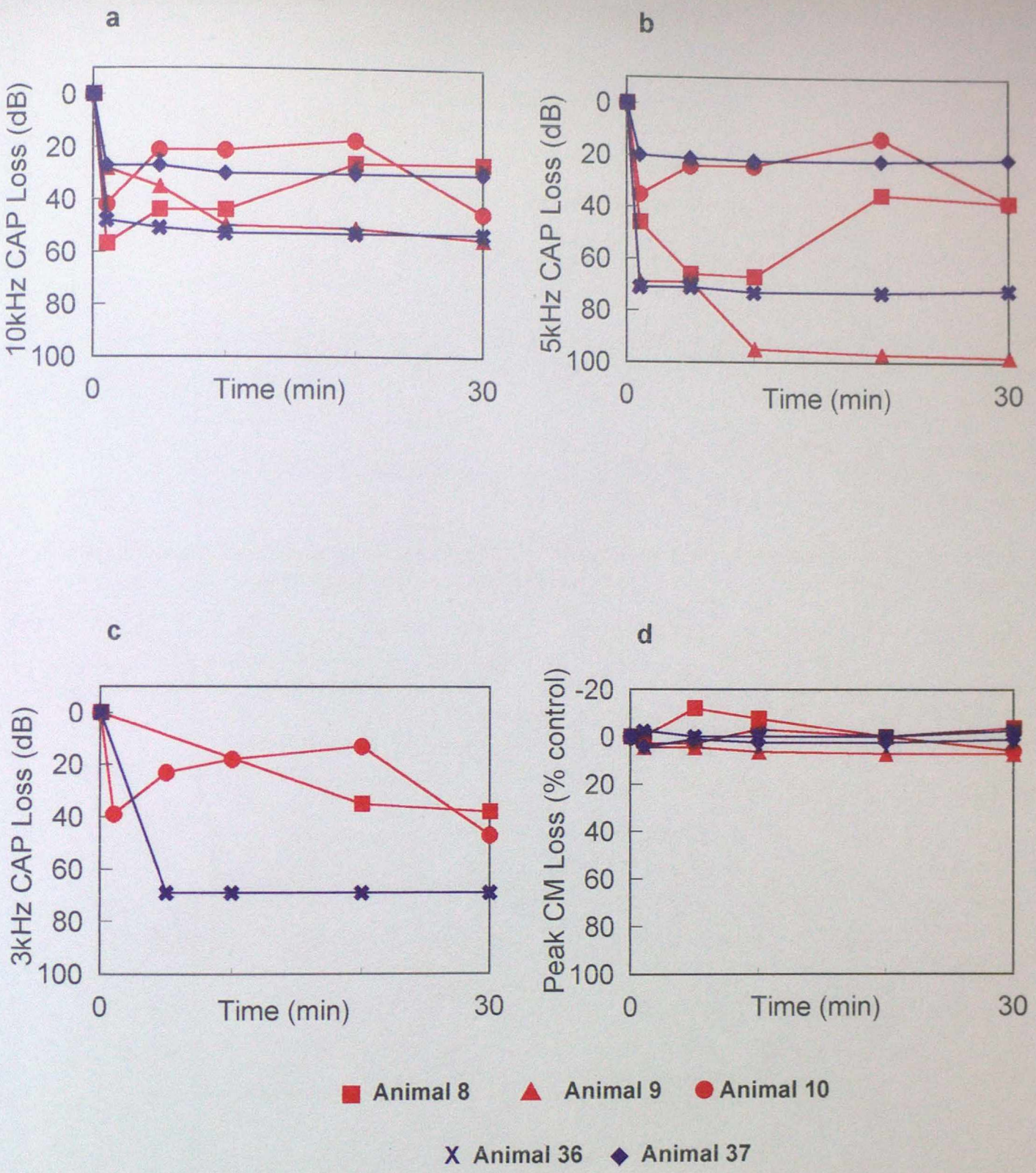


Figure 10.3.1.3. Losses in the CAP and CM in five animals treated with 10mM quisqualate, two pretreated with SOD (BLUE), and three not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

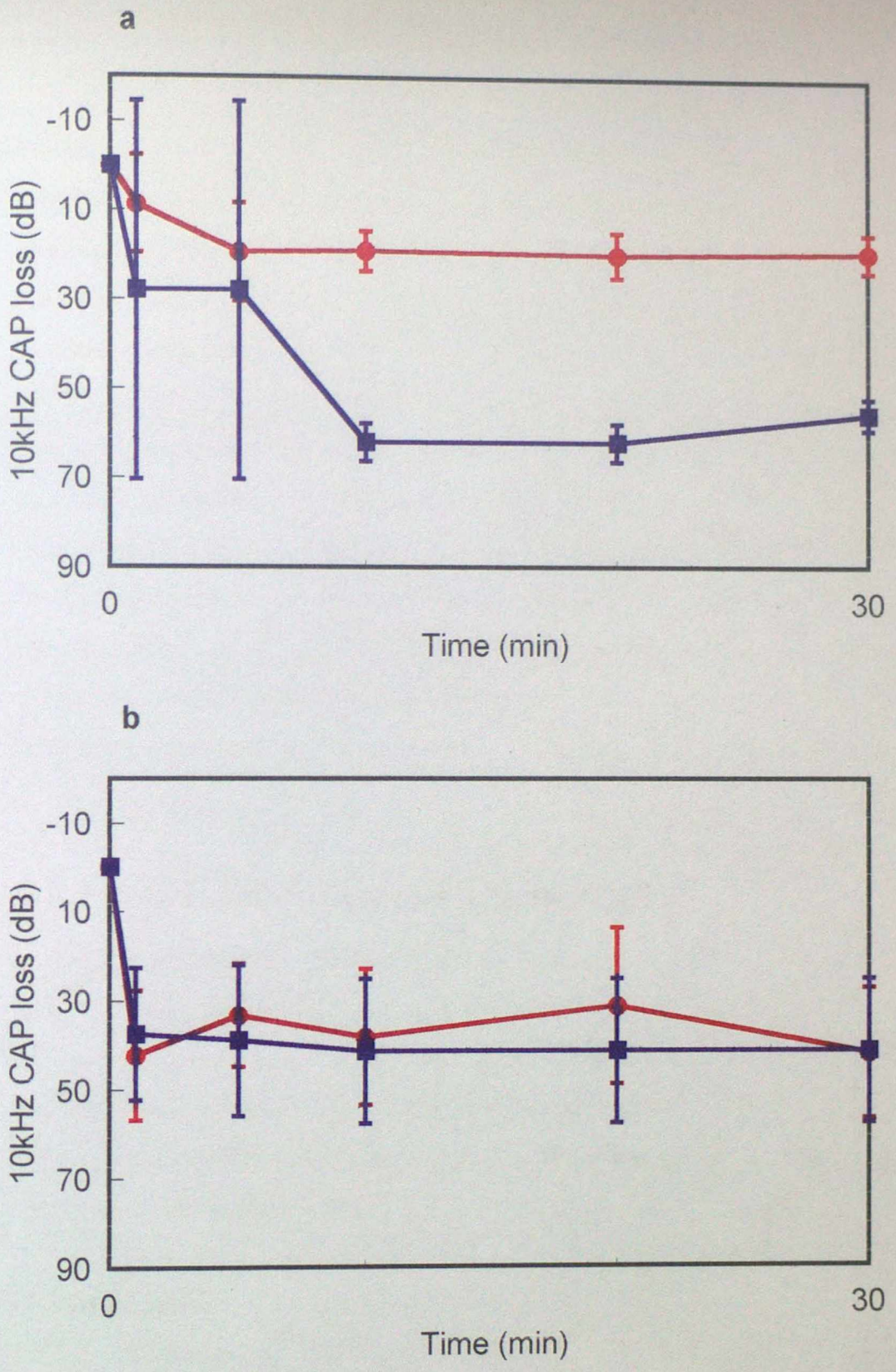


Figure 10.3.1.4. Average (± S.D) 10kHz CAP losses produced by perfusion of kainate and quisqualate with (■) and without(●) pretreament with SOD.

a) 100μM kainate b) 10mM quisqualate

Conclusions

- There was evidence of excitotoxicity produced by 100μM kainate and 10mM quisqualate despite pre-treatment of cochleae with SOD.
- Following perfusion with 100μM kainate, cochleae which had been pre-treated with SOD produced greater 10kHz CAP losses than non-pre-treated cochleae. Statistical analysis of the 10kHz CAP results by ANOVA demonstrated *almost* a significant difference between the data from SOD pre-treated and non-pre-treated cochleae perfused with 100μM kainate but not with 10mM quisqualate with respect to the effect of treatment on the CAP at all time points ($p= 0.05$ and 0.5 respectively). Neither set of data was significantly different with respect to the effect of treatment with time ($p= 0.3$ and 0.7 respectively).
- Therefore SOD did not protect cochleae from the excitotoxic effects of kainate and quisqualate at the concentrations used; pre-treatment with SOD actually worsened the excitotoxicity produced by 100μM kainate.

10.3.2 Pre-treatment with Deferoxamine (DEF)

1mM DEF was perfused into cochleae prior to perfusion of kainate and quisqualate in order to determine whether scavenging of the hydroxyl radical ($OH^{\bullet}$) protects cochleae against excitotoxicity produced by these non-NMDA agonists. DEF is a scavenger of the hydroxyl radical and has been shown to successfully protect the guinea pig cochlea from the ototoxic effect of excessive exposure to hydroxyl radicals at 1mM (Brechtelsbauer *et al.* 1994). Therefore 1mM DEF was used in this study.

10.3.2.1 DEF control

1mM DEF was perfused into cochleae of animals 38, 39, 40, 41, 42 and 43 **(Table 10.3.2.1)** in order to determine whether DEF itself produced any effects on cochleae potentials. No positive CAP or CM losses were displayed by any animal. At 10 min, 10kHz CAP losses were -9, 5, 5, -1, -20, and 6 dB in animals in animals 38-43 respectively. Animal 42 displayed increases in all CAPs

and CMs following perfusion which is indicative of poor initial coupling (as discussed in Section 3.1.). Nonetheless, there were no positive losses in CAPs or CMs when recalculated using the 1 min recordings as the control values.

Table 10.3.2.1 : Electrophysiological losses evoked by perfusion with 1mM deferoxamine in six animals. Positive losses are bold.

Time post perfusion (min)	Animal 38				Animal 39				Animal 40			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	10	15	31	0.18	20	0	35	0.18	35	25	41	0.17
	Loss in CAPs (dB) or CM (% control)											
1	-1	1	-1	-2	1	0	0	-4	1	3	2	0
5	-8	2	-1	2	1	11	5	-8	6	10	7	6
10	-9	2	2	2	5	17	7	2	5	11	5	17
30	-	-	-	-	1	17	6	10	-	-	-	-

Time post perfusion (min)	Animal 41				Animal 42				Animal 43			
	CAP			CM	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Threshold CAPs (dB) and Peak CM (mV)											
	37	39	50	0.17	24	29	43	0.15	38	21	42	0.11
	Loss in CAPs (dB) or CM (% control)											
1	1	1	1	2	-16	-24	-17	-19	6	7	4	-3
5	0	1	2	4	-21	-38	-23	-19	8	7	6	0
10	-1	1	0	2	-20	-39	-18	-28	6	7	5	-3
30	-	-	-	-	-	-	-	-	-	-	-	-

Conclusion

- There was no evidence of ototoxicity produced by 1mM DEF, therefore kainate and quisqualate were perfused into cochleae of these animals without any pre-existing electrophysiological damage having been caused by perfusion of DEF.

10.3.2.2 100μM Kainate following DEF Pre-treatment

100μM kainate was perfused into cochleae of animals 38 and 39 which had been pre-treated with DEF (**Table 10.3.2.2; Fig. 10.3.2.2**) and produced positive CAP losses in both animals. At 30 min, 10kHz CAP losses were 59 and 34 dB in animals 38 and 39 respectively. There were no positive CM losses.

Table 10.3.2.2 : Electrophysiological losses evoked by perfusion with 100μM kainate in two animals which had been pre-treated with 1mM DEF. Positive losses are bold.

Time post perfusion (min)	Animal 38				Animal 39			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post DEF)							
	1	17	33	0.17	21	17	41	0.16
	Loss in CAPs (dB) or CM (% control)							
1	**27**	7	-4	8	**24**	2	2	19
5	**58**	18	4	8	**47**	16	7	21
10	**58**	**20**	6	10	**47**	19	7	15
30	**59**	**22**	9	19	**34**	**21**	5	19

10.3.2.3 10mM Quisqualate following DEF Pre-treatment

10mM quisqualate was perfused into cochleae of animals 40, 41, 42 and 43 which had been pre-treated with DEF (**Table 10.3.2.3; Fig. 10.3.2.3**) and produced positive CAP losses in all animals. At 30 min, 10kHz CAP losses were 38, 59, 29 and 36 dB in animals 40-43 respectively. A positive CM loss of 93% was displayed by animal 41 only.

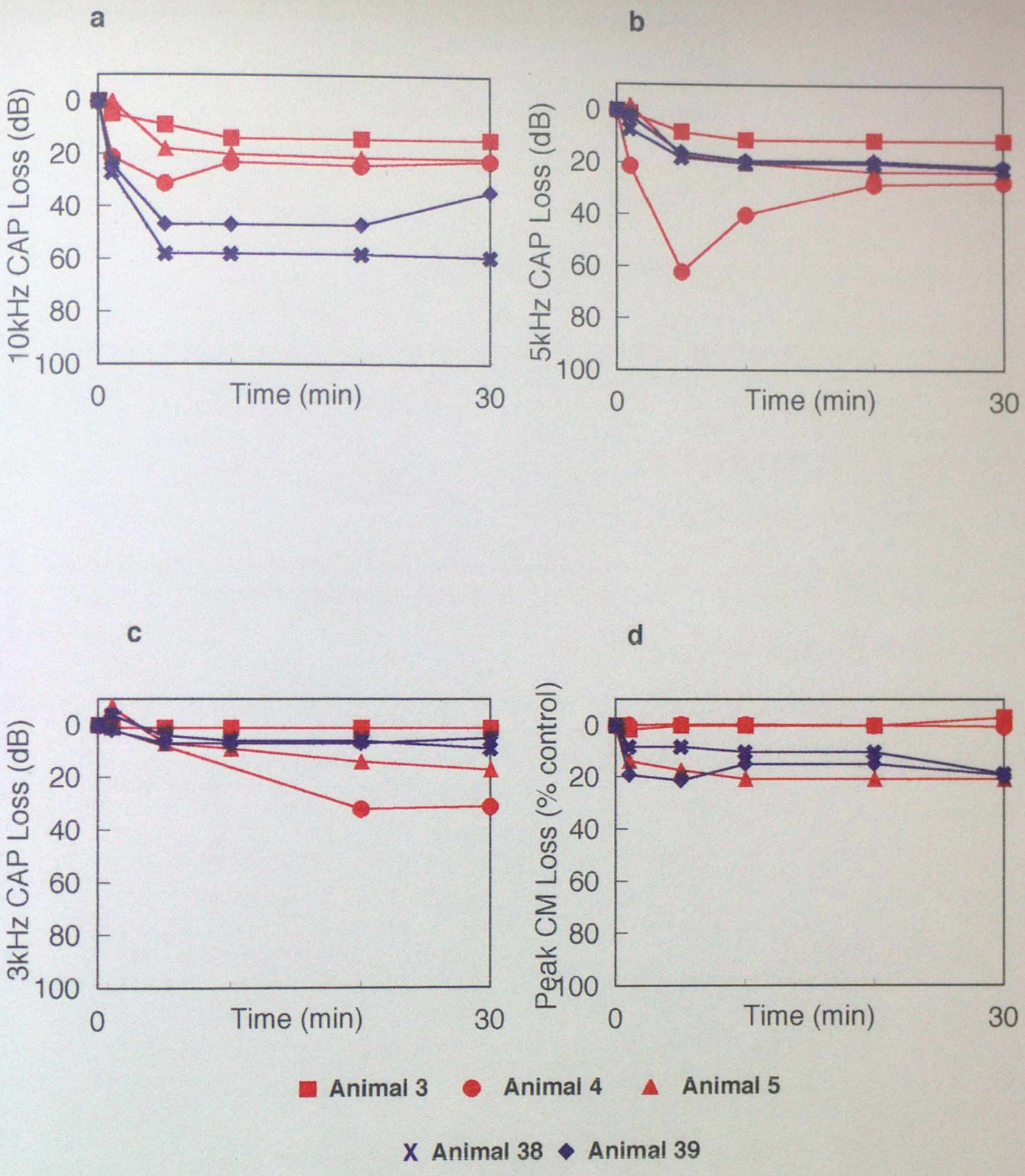


Figure 10.3.2.2. Losses in the CAP and CM in five animals treated with 100μM kainate, two pretreated with 1mM DEF (BLUE), and three not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

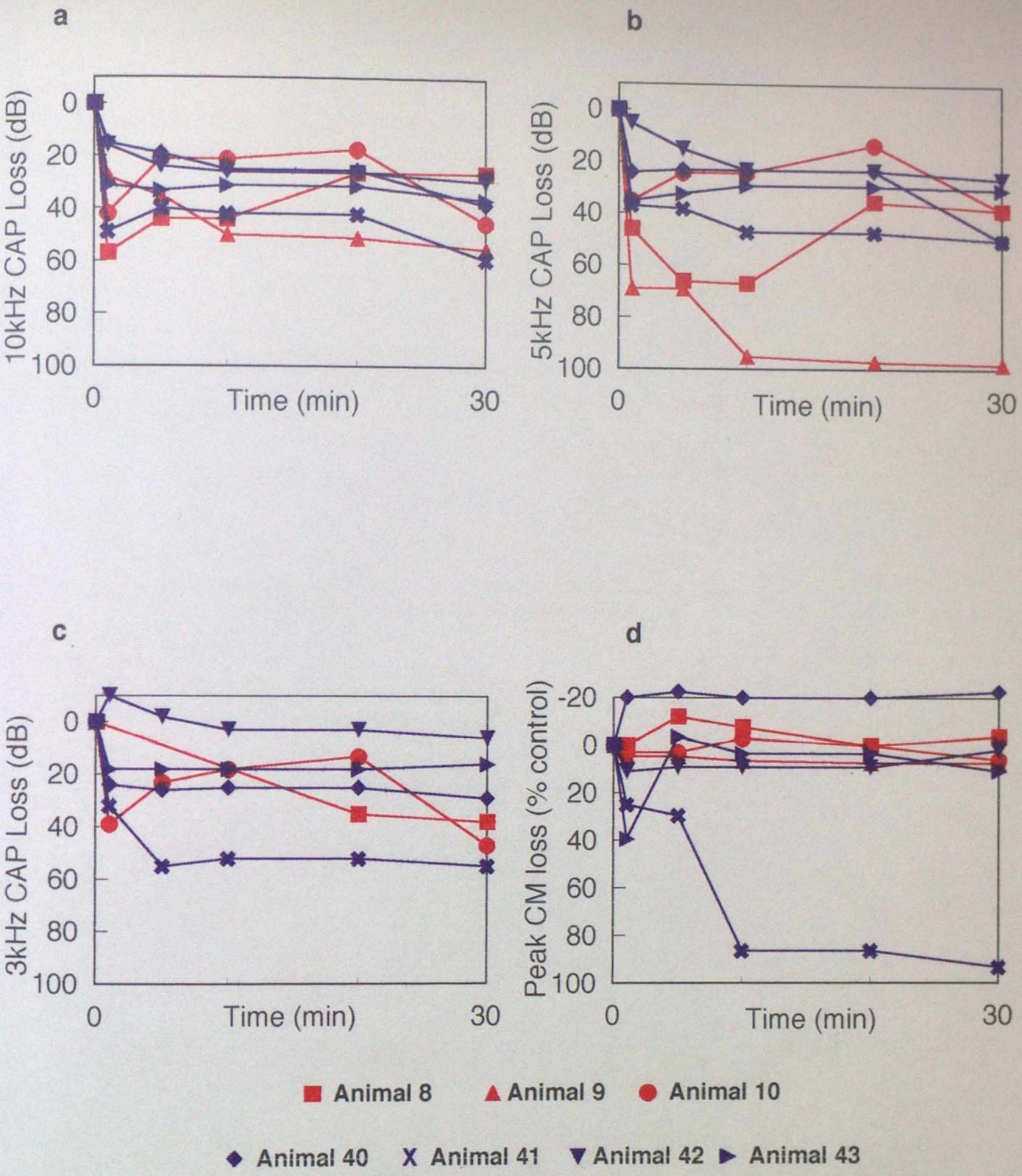


Figure 10.3.2.3. Losses in the CAP and CM in seven animals treated with 10mM quisqualate, four pretreated with DEF (BLUE), and three not pretreated (RED).

a-c) Losses in the CAP elicited with 10, 5 and 3kHz stimuli respectively. d) Loss in the CM elicited with a 5kHz stimuli.

Table 10.3.2.3 : Electrophysiological losses evoked by perfusion with 10mM quisqualate in four animals which had been pre-treated with 1mM DEF. Positive losses are bold.

Time post perfusion (min)	Animal 40				Animal 41			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post DEF)							
	40	36	46	0.14	36	40	50	0.17
	Loss in CAPs (dB) or CM (% control)							
1	15	**24**	**24**	-20	**49**	**37**	**32**	25
5	19	**23**	**26**	-23	**40**	**38**	**55**	29
10	**25**	**23**	**25**	-20	**42**	**47**	**52**	**86**
30	**38**	**50**	**29**	-23	**59**	**50**	**55**	**93**

Time post perfusion (min)	Animal 42				Animal 43			
	CAP			CM	CAP			CM
	10 kHz	5 kHz	3 kHz		10 kHz	5 kHz	3 kHz	
0 (control)	Pre-perfusion Threshold CAPs (dB) and Peak CM (mV) (i.e. post DEF)							
	14	-10	25	0.19	44	28	47	0.12
	Loss in CAPs (dB) or CM (% control)							
1	16	5	-10	11	**31**	**35**	18	39
5	**24**	15	-2	9	**33**	**32**	18	-4
10	**26**	**23**	3	9	**31**	**29**	18	3
30	**29**	**26**	6	2	**36**	**30**	16	11

10.3.2.4 Pre-treatment with DEF :Summary and Conclusions

30 min CAP and CM losses produce by kainate and quisqualate in cochleae with and without pre-treatment with DEF are summarised in **Fig. 10.3.2.4** and **Table 10.3.2.4.**

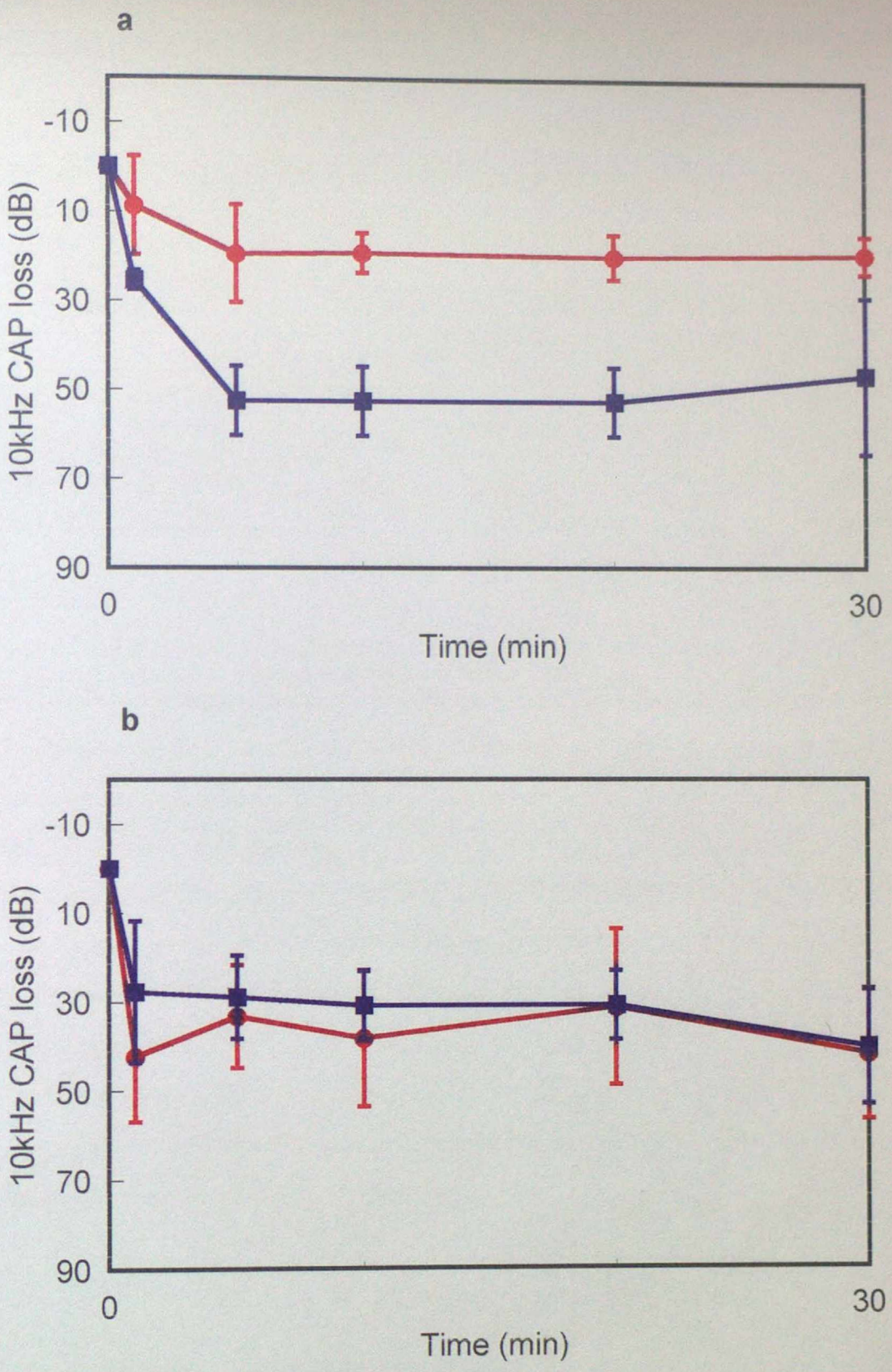


Figure 10.3.2.4. Average (± S.D) 10kHz CAP losses produced by perfusion of kainate and quisqualate with (■) and without(●) pretreament with 1mM DEF.

a) 100μM kainate b) 10mM quisqualate

Table 10.3.2.4 : Average 30 min losses in CAPs and CMs produced by perfusion with 100μM kainate (KA) and 10mM quisqualate (QA) with or without prior perfusion of 1mM DEF (+/- respectively). Positive losses are bold.

Treatment	Average losses (± SD) in CAPs (dB) or CM (%) at 30 min post perfusion			
	10 kHz CAP	5 kHz CAP	3kHz CAP	CM
100μM KA - DEF (n=3)	19 ± 4	**20 ± 8**	16 ± 5	6 ± 13
100μM KA + DEF (n=2)	**46 ± 18**	**22 ± 1**	7 ± 3	19 ± 0
10mM QA - DEF (n=3)	**42 ± 15**	**58 ± 35**	**42 ± 6**	3 ± 6
10mM QA + DEF (n=4)	**42 ± 16**	**46 ± 36**	**69**	-1 ± 3

Conclusions

- There was evidence of excitotoxicity produced by 100μM kainate and 10mM quisqualate despite pre-treatment of cochleae with DEF.
- Statistical analysis of the 10kHz CAP results by ANOVA demonstrated a significant difference between the data from DEF pre-treated and non-pre-treated cochleae perfused with 100μM kainate (0.0231, 0.1149), but not those perfused with 10mM quisqualate with respect to treatment alone (p= 0.02 and 0.8 respectively). Neither set of data was significantly different with respect to the change in the CAP with time (p= 0.1 and 0.9 respectively). Following perfusion with 100μM kainate, cochleae which had been pre-treated with DEF produced statistically greater 10kHz CAP losses than non-pre-treated cochleae.
- Pre-treatment with DEF provided no protection on cochleae from the effects of kainate and quisqualate at the concentrations used. However, pre-treatment with DEF actually worsened the excitotoxicity produced by 100μM kainate.

10.3.3. Morphological Examinations

10.3.3.1. 100μM Kainate following SOD or DEF pre-treatment

Cochleae pre-treated with SOD or DEF and perfused with 100μM kainate revealed more extensive afferent nerve terminal damage than seen in cochleae that had not been pre-treated. In addition to mitochondrial and membrane damage of the type I afferents (seen in non-pre-treated

cochleae), some afferent nerve terminals beneath the inner hair cells were swollen and denuded of cellular contents.

10.3.3.2. 10mM Quisqualate following SOD or DEF pre-treatment

Cochleae pre-treated with SOD or DEF and perfused with 10mM quisqualate revealed widespread damage to the afferent nerve terminals at the base of the inner hair cell with some associated damage to surrounding structures, very similar to that seen in non-pre-treated cochleae.

Conclusion

There was no morphological evidence that SOD or DEF afforded any protection on cochleae from the excitotoxic action of either quisqualate or kainate at the concentrations used. Moreover, SOD and DEF actually worsened the afferent nerve terminal damage of inner hair cells produced by 100μM kainate.

10.3.4. Effect Of Pre-treatment With Free Radical Scavengers On The Degree Of Excitotoxicity Produced By Kainate And Quisqualate : Summary And Conclusions

30 min 10kHz CAP losses produced by perfusion of kainate and quisqualate in cochleae with and without pre-treatment with free radical scavengers are summarised in **Fig. 10.3.4 a**.

Conclusions

- At the concentrations used, neither SOD nor DEF afforded any protection on cochleae from the excitotoxic action of either kainate or quisqualate as judged by electrophysiological and morphological criteria. Both SOD and DEF apparently enhanced the excitotoxic action of 100μM kainate.
- The results could be interpreted in several ways.

  (i) Neither SOD nor DEF were active in cochleae in terms of scavenging for superoxide and hydroxyl radicals. However, the data from pre-treated cochleae were statistically

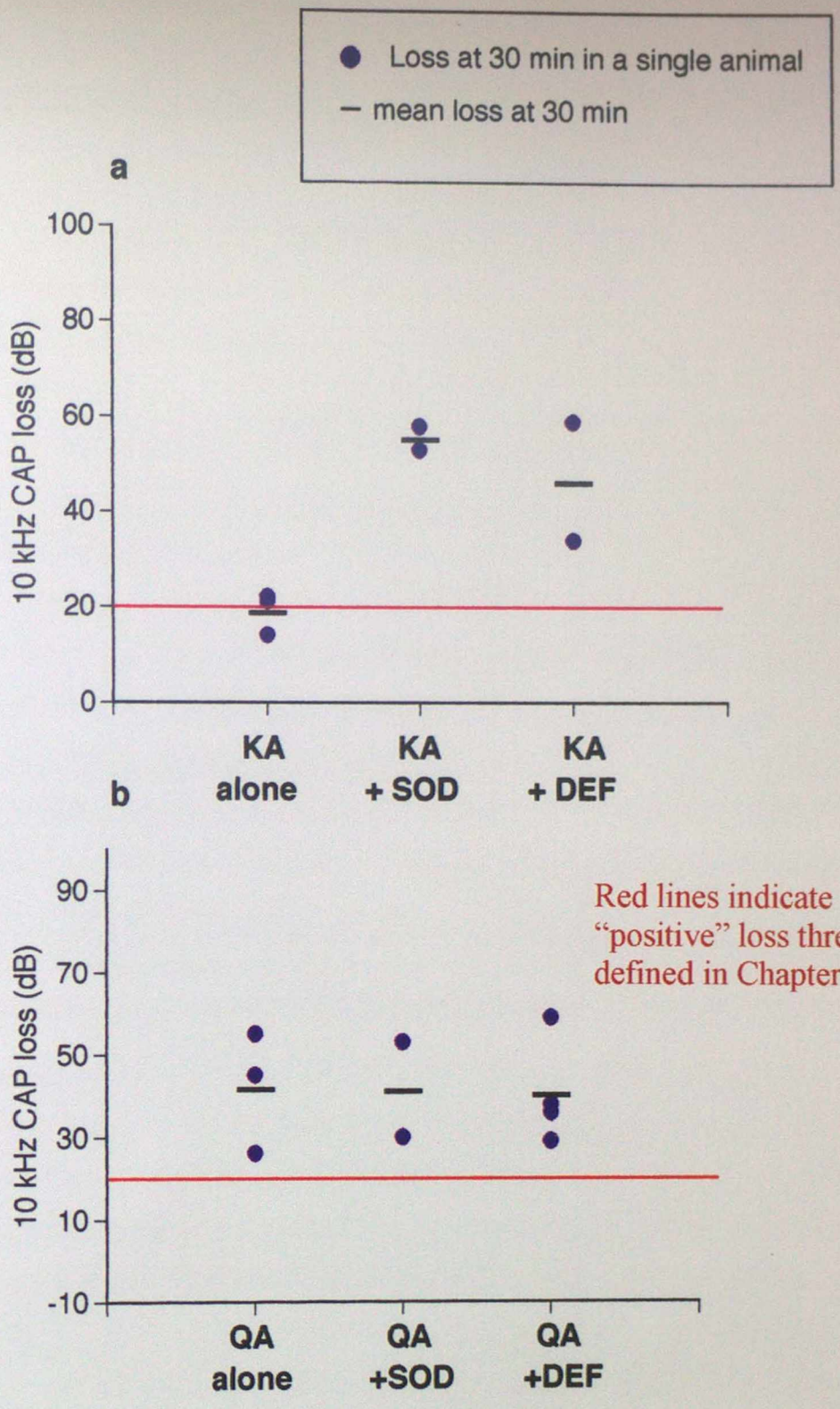


Figure 10.3.4.a **30 min 10kHz CAP losses produced by perfusion of kainate (KA) and quisqualate (QA) with and without pretreament with SOD and DEF.**

a) 10mM kainate perfusions b) 100μM kainate perfusions

different than the data from non-pre-treated controls, therefore it appears that the inhibitors were having some effect within cochleae. In addition, SOD and DEF had previously been shown to be active within cochleae in inhibiting the effects of $O_2^{\bullet}$ and $OH^{\bullet}$ generation systems.

(ii) SOD and DEF may have produced some ototoxicity themselves beyond the 10-30 min recordings that were taken in control experiments. However, SOD (50unitsml^{-1}) and DEF (1mM) had been previously shown to produce no CAP losses up to 120 min post perfusion when they were perfused into cochleae in combination with SOD and DEF generating systems (Clerici and Yang 1996). Also cochleae perfused with NMDA which had been pre-treated with SOD (1560unitsμl^{-1}) produced no CAP losses up to 90 min post perfusion (Amaee *et al.* 1997). Moreover, the timing of the losses in this chapter and morphological evidence is characteristic of losses associated with kainate and quisqualate.

(iii) Another consideration to be made is that the protective action of these scavengers reported by Clerici and Yang (1996) was seen in CAPs measured at frequencies between 9 and 30kHz. Therefore any protective action of SOD and DEF on the action of kainate and quisqualate in my study might have been evident at higher frequencies. However, partial data from incomplete experiments carried out for this thesis concerning CAP at losses at 20kHz (**Fig. 10.3.4 b**) did not support this. Perfusion with kainate and quisqualate in animals pre-treated with SOD or DEF produced CAP losses at 20kHz which were greater than or equal to the losses in animals untreated or treated with L-MA; the latter showed no protection at other frequencies. However, further data are needed to draw firm conclusions.

(iv) The most likely interpretation of the data concerning ROSs is that superoxide and the hydroxyl radical are not involved in mediating the excitotoxic action of kainate and quisqualate. However, since scavenging these radicals worsened the excitotoxicity produced by kainate and quisqualate, it is just possible that superoxide and hydroxyl radicals may play a protective role in the excitotoxicity mediated by these non-NMDA agonists in cochleae. This was not further examined.

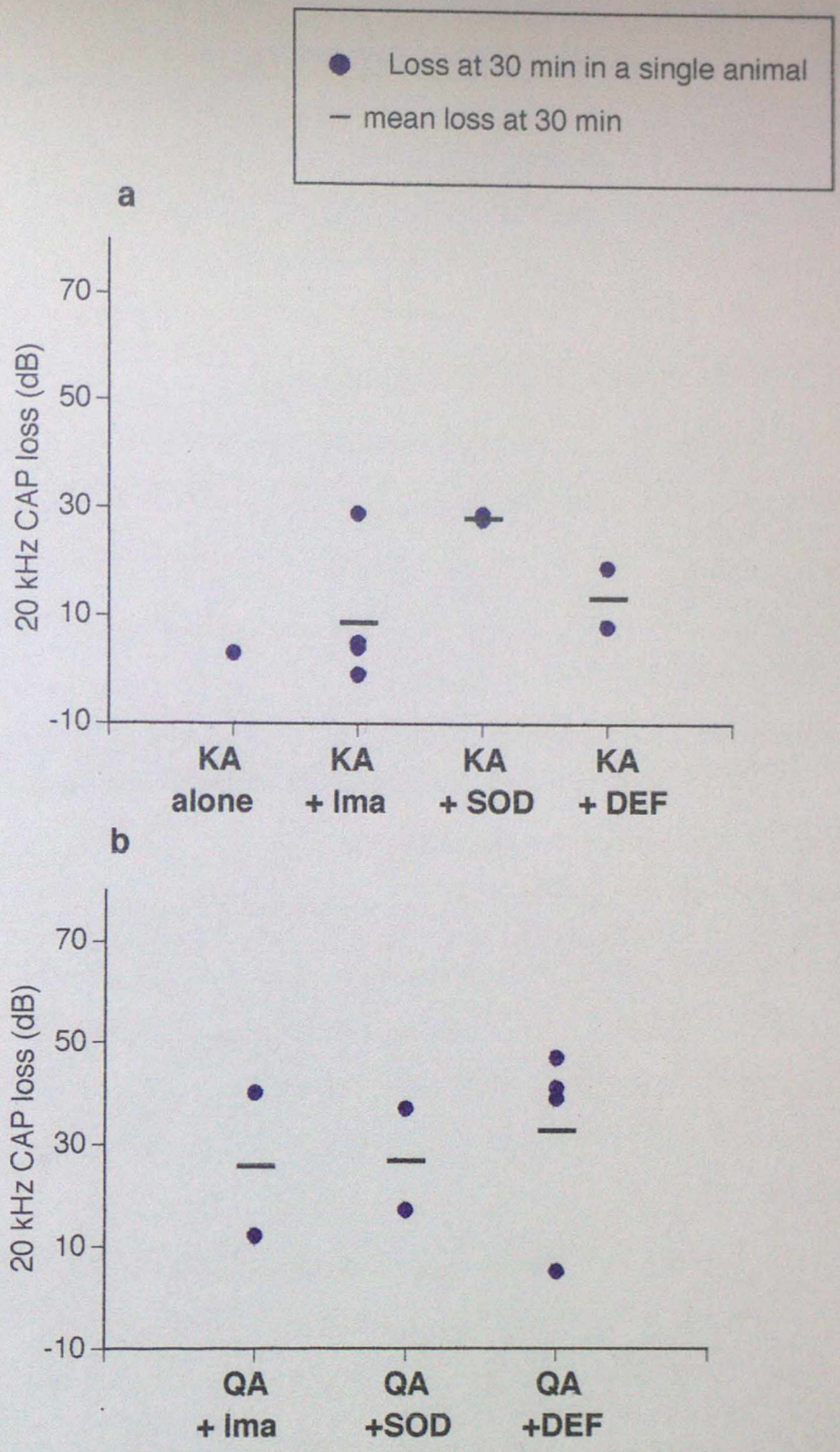


Figure 10.3.4.b. **30 min CAP losses at 20kHz produced by perfusion of kainate (KA) and quisqualate (QA) with and without pretreament with L-MA (lma) , SOD or DEF.**

a) 100μM kainate perfusions b) 10mM quisqualate perfusions

CHAPTER 11
Discussion

In this concluding chapter I shall discuss the results in Chapters 3-10, the validity of the techniques used, and further confirmatory experiments which should be carried out. Finally I shall discuss the most likely causes of meningitis-induced deafness, the most likely preventative measures and the direction in which I feel future work should be directed.

11.1. Summary of the Results

1. The evidence presented confirmed the presence of a potent ototoxic protein(s) in cytoplasmic extracts of a clinical isolate of *H. influenzae type b*.

2. The evidence presented also suggests that there may be present in low concentration an ototoxin in cytoplasmic extracts of a clinical isolate of *N. meningitidis*, further confirmatory investigations are clearly needed.

3. There was evidence for an ototoxic factor in the cytoplasmic extracts of a pneumolysin-deficient mutant of *S. pneumoniae type III* which may represent a novel ototoxin. A similar ototoxin in the bacterial extract of wild type *S. pneumoniae type III* may have been responsible for the ototoxicity produced by this extract after immunochemical removal of pneumolysin. However the presence of some contaminants in the immunopurified extract, makes this conclusion tentative.

4. There was evidence of 'pneumolysin-like' proteins in the bacterial extracts of *N. meningitidis* and *H. influenzae type b* similar to that present in extracts of wild type *S. pneumoniae type III.* The relative concentration of these proteins was greatest in pneumococcal extracts, and less in *H. influenzae type b* extracts and considerably less in a meningococcal extract. However, further confirmatory experiments are necessary in order to eliminate the role of cross-track contamination in these findings.

5. Pre-treatment of guinea pig cochleae with L-NAME, L-MA, SOD or DEF provided no protection against the excitotoxicity produced by kainate and quisqualate at the concentrations used.

11.2. Novel ototoxins/ototoxic factors in *H. influenzae type b, N. meningitidis* and *S. pneumoniae type III* bacterial extracts

In this thesis evidence is adduced for the existence of novel ototoxins/ototoxic factors in bacterial extracts of *N. meningitidis, H. influenzae type b,* and *S. pneumoniae type III.* However further investigations are necessary, particularly with regards to *N. meningitidis.* This is the first time to my knowledge that evidence has been produced for the existence of directly acting ototoxic factors in bacterial extracts of *H. influenzae type b, N. meningitidis* and pneumolysin-deficient *S. pneumoniae type III.* The ototoxicity produced by these bacterial extracts is most likely to have been due to the presence of hitherto undescribed ototoxic proteins for the following reasons. First, low molecular ototoxic species (e.g. NO, glutamate and hydrogen peroxide) would have been eliminated by dialysis. Second the time course of CAP losses was in general consistent with hearing losses being produced by direct ototoxicity and not by secondary ototoxic events mediated by the inflammatory response. Third, those fractions of *H. influenzae type b* and meningococcal bacterial extracts which were likely to have contained large amounts of endotoxin were either not

as ototoxic as other fractions, or, not ototoxic at all. Fourth, some bacterial extracts were not ototoxic as for example pneumococcal extracts when not DTT-treated. Therefore, ototoxicity was not due to non-specific effects of perfusing a crude bacterial extract into the cochlea such as the increased viscosity of the perilymphatic fluid. Finally, ototoxicity produced by those bacterial extracts examined was specific as judged by electrophysiological measurements and morphological examinations.

The degree of ototoxicity produced by each bacterial extract reflected the relative concentrations of 'pneumolysin-like' proteins in each extract, i.e. CAP losses produced were greatest with pneumococcal extracts, less with *H. influenzae type b* extracts, and least with meningococcal extracts. Therefore it is conceivable that these 'pneumolysin-like' proteins were responsible for the ototoxicity produced in guinea pig cochleae. However, further studies will be necessary to confirm or weaken these conclusions. If they were confirmed, it would be the first time to my knowledge that, SH-activated ototoxins had been demonstrated in the two gram negative bacteria *H. influenzae type b* and *N. meningitidis. If* these 'pneumolysin-like' proteins in meningococci and *H. influenzae type b* are ototoxins which are thiol-activated and released by autolysis similar to pneumolysin, this would explain the *lack* of ototoxicity of culture *filtrates* and *increased* activity of the crude extracts after *DTT treatment.*

The ototoxicity produced by the bacterial extracts was comparable with that produced by live bacteria in the cochlea following direct perfusion or experimental meningitis (see next section). Therefore the ototoxic factors in the crude extracts of these bacteria may be responsible for the hearing losses associated with these pathogens. In fact, both the relative ototoxic potency (as judged by CAP losses at 90 min) and the relative concentration of 'pneumolysin-like' proteins in these bacterial extracts seem to correlate with the incidence of meningitis-induced deafness associated with these pathogens (see **Section 1.2.**). Moreover, the incidence of hearing loss associated with *N. meningitidis type b* may be less than that reported for *N. meningitidis* (of all

serotypes) because it has been reported that the type b serotype causes considerably fewer cases of deafness than other serotypes (Mayatepek, Grauer, Hansch *et al.* 1993).

Although the main aim of this study was to identify and isolate ototoxins in the bacterial extracts of the three main pathogens responsible for bacterial meningitis, attention was diverted to characterisation of the mode of ototoxic action by establishing the type of electrophysiological losses produced and ultrastructural changes by examination of the cochlea.

11.2.1. Characterisation of Novel "Ototoxins"

Electrophysiological recordings revealed that ototoxicity produced by *H. influenzae type b*, meningococcal and pneumococcal bacterial extracts consistently included a fall in the CAP but not the CM. Any CM losses produced were mostly associated with losses in the CAP of >40dB. Losses in CAP were apparent across several frequencies. Two types of time course of ototoxicity were produced by the bacterial extracts described here. Ototoxicity produced by *H. influenzae type b* extracts and pneumococcal extracts containing pneumolysin were usually rapid, the majority of the CAP loss being achieved within 10 min. Meningococcal extracts and pneumococcal extracts which did not contain free pneumolysin produced ototoxicity whose onset was a little slower, the majority of the loss occurring after 20 or 30 min.

The electrophysiological evidence implied that the major target of all bacterial ototoxic factors described here was the afferent neuron, afferent synapse or inner hair cell, and these factors were uniformly active along the length of the cochlea. In contrast, outer hair cells were mostly unaffected. The time courses of all hearing losses described here were sufficiently rapid to imply that these bacterial extracts were directly toxic to the cochlea and did not produce ototoxicity by evoking an inflammatory response.

SEM examination of treated cochleae was often difficult due to curling of the specimens which appeared to be affected by perfusion of the extract, because it was not apparent in similarly treated control cochleae. Nevertheless, the ototoxicity produced by all bacterial extracts was associated with some evidence of structural damage to the surface of the organ of Corti. There was some damage to the stereocilia of inner and outer hair cells (splaying, detachment and fusing), and some swelling and cratering of supporting cells, particularly the border cells. Stereocilial damage was commonly co-located with craters in supporting cells and more often affected inner hair cells. However, with the possible exception of wild type *S. pneumoniae* extract the surface structural damage produced by all extracts was very patchy, inconsistent between animals, and was not always correlated with electrophysiological losses. SEM examination of cochleae treated with wild type pneumococcal extract containing free pneumolysin revealed stereocilial damage and cratering in the border cells which was less patchy than seen in cochleae treated with other bacterial extracts. In addition there was striking swellings of the supporting cells between outer hair cells. However, examination was only possible in one cochlea due to curling. The apparent discrepancy between electrophysiological losses and morphological damage was resolved by TEM examination which revealed consistent morphological damage, the degree of which correlated with electrophysiological losses in all cochleae examined in this way. Ultrastructural examination by TEM revealed consistent and striking damage to the mitochondria of nerve endings at the base of the hair cells, particularly the inner hair cells. In cochleae which had displayed large CM losses, severe mitochondrial damage was apparent in all cells including the inner and outer hair cells. Cochleae treated with meningococcal extracts, and pneumococcal extracts which did not contain free pneumolysin were not examined by TEM due to either lack of specimens, or lack of time (see **Section 2.2.5.**). Since the surface structural damage produced by these extracts was not consistent with electrophysiological losses, it is most likely that ultrastructural damage was responsible for the electrophysiological losses which cannot be seen by SEM and would have been seen by TEM.

Thus, all cochleae which showed electrophysiological losses, consistently revealed ultrastructural damage observed following examination by TEM but not by SEM. Therefore it seems likely that the primary action of the bacterial extracts was most likely to have been to the under side of the organ of Corti, in particular the mitochondria of the nerve endings. Surface structural damage was most likely secondary to this ultrastructural damage to the nerve endings and hair cells and so was less consistently produced.

11.2.2. Comparisons with Previous Studies

The electrophysiological and morphological data were in general consistent with previous preliminary studies (as discussed previously in Chapters 4 and 8). However, there were some discrepancies with respect to pneumococcal extracts, but these probably arose from differences in protein concentrations and DTT treatment. Apart from these preliminary studies, there have been no other studies which involved investigating the ototoxicity of crude bacterial extracts. There has however, been some previous investigations carried out into the effects of purified toxins and live bacteria in the cochlea which can be compared with the data described here. Several groups have evaluated hearing losses and carried out morphological examinations of the cochlea following experimental meningitis. However, since the only electron microscopic examinations and direct cochlear recordings have been carried out in our laboratories I shall limit this discussion to data from previous experiments within our laboratories as they are most comparable to the data presented here.

H. influenzae type b "ototoxin"

The ultrastructural damage to the organ of Corti described here following perfusion of *H. influenzae type b* bacterial extracts was also reported following SEM and TEM examination of cochleae following *experimental meningitis in rabbits induced by H. influenzae type b* (Osborne *et al.* 1995). However, experimental meningitis produced some additional structural damage such

as pyknosis of hair cell nuclei, swollen or empty nerve endings, vacuolation of cells within stria vascularis and some evidence of intracochlear bleeding. The hearing losses produced by experimental *H. influenzae type b* meningitis were measured by click evoked ABERs and so are not directly comparable with the CAP data reported here. Since loss of sensitivity to click stimuli represents more profound hearing losses than equivalent losses to tone pip stimuli, the 9-50dB losses detected by ABERs (over 9-20h) are probably similar if not greater than the losses produced by *H. influenzae type b* "ototoxin" described here. There are no reports of direct cochlear recordings during experimental *H. influenzae type b* meningitis.

Wild type pneumococcal 'ototoxins'

The ototoxicity produced by wild type pneumococcal extract containing free pneumolysin is almost identical to that produced by *live pneumococci (type II) perfused directly into the cochlea* in terms of morphological damage examined by SEM, and CAP and CM losses. However, the onset of CAP losses produced by live pneumococci was delayed in comparison to bacterial extracts. *Experimental pneumococcal (type II) meningitis in guinea pigs* produced more extensive cochlear damage (as judged by both electrophysiological recordings and TEM and SEM examinations) than cochlear perfusion with bacterial extracts or live pneumococci. Following experimental pneumococcal meningitis, cochleae revealed all the structural damage sustained by perfusion of wild type pneumococcal extract, or live wild type pneumococci. However additional damage was seen including severe outer hair cell and supporting cell damage (swollen and vacuolated, breaks in basal and apical membranes and mitochondrial vacuolation), and disruption of intercellular tight junctions (Winter *et al.* 1997). In addition, experimental pneumococcal meningitis often produced large CM losses, whereas perfusion of pneumococcal extracts and live pneumococci did not. The effects of *perfusing purified pneumolysin into the cochlea* were earlier described as very similar to those described following experimental pneumococcal meningitis (as judged by CAP and CM losses and SEM examinations). Similar but more extensive surface

structural damage was revealed than that produced by perfusion of wild type *S. pneumoniae* extract (containing free pneumolysin), or live wild type pneumococci. Additional pitting, swelling and cratering of the hair cells was seen, and large CM losses were produced in addition to CAP losses (Comis *et al.* 1993).

New (?) pneumococcal ototoxin

Cochleae treated with pneumococcal extracts which did not contain free pneumolysin revealed very similar surface structural damage to that described following *experimental meningitis induced by pneumolysin-deficient pneumococci* (type II) (as judged by CAP and CM losses and SEM examinations). Some CAP losses were observed, but smaller than those produced by experimental wild type pneumococcal meningitis. CM losses and surface structural damage were not consistently produced by the pneumolysin-deficient mutants. TEM examination following experimental meningitis induced by pneumolysin-deficient pneumococci revealed consistent vacuolation of mitochondria in the nerve endings (Winter *et al.* 1997). Similar ultrastructural damage may have been responsible for the CAP losses produced by pneumococcal extract which did not contain pneumolysin, since surface structural damage was not consistent.

The greater extent of cochlear damage produced by pneumolysin in comparison to pneumococcal extracts and live pneumococci following cochlear perfusion is most likely to have been due to a difference in the concentration of pneumolysin present. 1-10μg of pneumolysin produces large CAP and CM losses, whereas 0.5μg pneumolysin produces only transient and cumulative CAP losses with no CM loss within 90 min (Comis *et al.* 1993). Therefore, either an additional ototoxin (perhaps similar to that responsible for ototoxicity in both the pneumococcal extracts which did not contain free pneumolysin) combined with a small concentration of pneumolysin produced the CAP losses observed. Or alternatively, since the effects of pneumolysin have only been examined over 90 min previously, a small concentration of pneumolysin may lead to permanent CAP but not

CM losses if present in the cochlea for greater than 90 min. The delay of onset of CAP losses produced by live bacteria in comparison to bacterial extracts is likely to represent the time that it took pneumococci to release a sufficient amount of pneumolysin (and any other ototoxins) by multiplication and autolysis to cause ototoxicity.

Cochlear damage produced by experimental meningitis compared with cochlear perfusion

It is of interest to ask whether the damage seen in experimental meningitis is comparable to that seen in cochlear perfusion. As far as the work of this group is concerned, the most ***direct*** comparison that could be made of the nature and extent of damage caused by these two routes involved experiments with the relatively non-pathogenic *E. coli* K12. A greater degree of cochlear damage was produced by experimental meningitis than by cochlear perfusion. *E. coli* K12 produced no significant electrophysiolgical losses or ultrastructural damage to the cochlea when perfused directly into the cochlea (Winter 1997), yet produced considerable hearing losses (as judged by ABERs) and ultrastructural damage in an experimental meningitis model (Osborne *et al.* 1995); there are several explanations for these differences. First, the time course of experimental meningitis is longer and so may provide a longer exposure of the cochlea to bacterial components/toxins and inflammatory mediators. Second, the higher total number of bacteria arising from a longer experimental time, could result in the generation/release of a greater amount of bacterial components and inflammatory mediators. Third, complications of the inflammatory response in the CSF such as oedema may have caused some ototoxicity (e.g. ototoxicity induced by ischaemia). Finally, perforated cochleae may be less sensitive to ototoxic agents than intact cochleae.

11.2.3. Use of the Cochlear Perfusion Model in Identifying and Isolating Bacterial Ototoxins

The data presented here suggest that electrophysiological and morphological examination of cochlea following perfusion of bacterial extracts can provide valuable insight into the type of ototoxicity produced by bacterial pathogens. This method may not be able to detect hearing losses that are the cumulative result of inflammatory responses in the CSF, but is able to identify bacterial components which are directly ototoxic as opposed to those which only produce ototoxicity through the inflammatory responses. My results suggest that identification and isolation of ototoxins from meningogenic pathogens is possible using the cochlear perfusion model as a screening/detection system. However, the cost and inter-animal variation associated with the use of guinea pigs makes isolation of ototoxins a time consuming and expensive task, especially if the ototoxic potency of the crude extract is low. For example, the results presented in section 4 were carried out using one batch of crude *H. influenzae type b* bacterial disruptate received from CAMAR. Subsequent batches of crude *H. influenzae type b* bacterial disruptate yielded less reproducible results and displayed mild ototoxicity in comparison to the data presented here. It is thus possible that the toxin may be labile in preparation or under-expressed under certain growth conditions. Expression of ototoxin by this strain of *H. influenzae type b* may have been lost or reduced by repeated *in vitro* growth of the organism. If the ototoxin is a virulence factor, passage of the bacterium through an animal could restore its production. Alternatively, new clinical isolates could be used, in particular strains that were responsible for causing deafness. However, the incidence of hearing loss in experimental meningitis induced by *H. influenzae type b* was not greater in animals which had been infected with strains which had caused clinical deafness (Kaplan *et al.* 1989). The efficiency of the disruption process could be monitored in terms of protein concentrations to ensure comparable concentration of proteins in each batch. The degree of disruption could also be monitored by assaying the concentration of succinate dehydrogenase (a membrane bound enzyme found on the inner surface of plasma membranes). DTT treatment (and/or using degassed APL) should be considered for all extracts because the ototoxicities of bacterial extracts of *S. pneumoniae* and *H. influenzae type b* (including later batches of *H.*

influenzae type b extract not described in this thesis) were enhanced by DTT treatment of the sample. Therefore, by monitoring the growth and disruption, and preparation of the extracts, methods could be optimised to produce maximally potent bacterial extracts.

Future experiments should focus on production of high activity bacterial extracts, and developing better methods for fractionating the ototoxic factors in these bacterial extracts. In addition, the culture filtrate of meningococcus should be tested for ototoxicity to see if any secreted ototoxins were present. Once ototoxins have been isolated, amino acid sequence could be determined, the gene/s responsible for their production identified which would permit all kinds of sophisticated molecular biological experiments to be considered.

11.3. Excitotoxicity produced by quisqualate and kainate in the cochlea

11.3.1. Excitotoxicity produced by quisqualate and kainate in the cochlea

Perfusion of 10mM quisqualate or 10mM kainate into the cochlea produced large losses in CAPs, but not CMs. Transmission electron microscopy revealed widespread swelling of the afferent nerve endings of the inner hair cells with some associated damage to the base of the inner hair cells and supporting cells, particularly in cochleae treated with kainate. There was no evidence of damage to the outer hair cells, although the cytoplasmic contents of the efferent nerve endings at the base of the outer hair cells appeared to be more dense than in controls. 100μM kainate produced small CAP losses which were associated with a small degree of mitochondrial and membrane damage in the afferent neurons at the base of the inner hair cell. In addition there was some fragmentation of the plasma membrane at the base of the inner hair cell.

11.3.1.1. Comparisons with previous data

As previously discussed (Chapter 10), the results presented here are consistent with the previous data concerning the action of quisqualate and kainate in the cochlea (Jenison *et al.* 1986; Bledsoe *et al.* 1981; Pujol *et al.* 1985). The CAP losses are likely to be a consequence of an initial increase in the spontaneous activity (excitation) followed by a post excitatory depression (excitotoxicity) of the type I afferents which is produced by perfusion of quisqualate and kainate into guinea pig cochleae (Bledsoe *et al.* 1981; Jenison and Bobbin 1985). The slight structural damage produced by 100μM kainate and the increase in density of the efferent neurons beneath the outer hair cells has not been previously reported. In addition, there have been no previous reports of ultrastructural examinations following quisqualate mediated excitotoxicity in the cochlea.

The function of the medial efferents is thought to be to modulate the motile response of outer hair cells. Therefore medial efferents could alter the stimulus for the inner hair cells by modifying the amplification of the basilar membrane movement produced by outer hair cell motility. The increase in the density of the medial efferents beneath the outer hair cells could represent a change in the activity of the efferent neurone in response to either the initial excess activation, or the post-excitatory depression of activity of the type I afferents. However, there was no detectable change in the CM during excitotoxicity produced by kainate, quisqualate or AMPA (Bledsoe *et al.* 1981; Jenison *et al.* 1986; Puel *et al.* 1991), nor in OAEs during excitotoxicity produced by kainate (Zheng, Wang, Salvi *et al.* 1996). Therefore, either efferents can produce changes in the motility of OHCs without affecting CMs and OAEs, or there was some change in the cytoplasm of the medial efferent dendrites which did not affect the function of the outer hair cells.

The slight structural damage produced by 100μM kainate may represent the initial effect of kainate on the type I afferent nerve endings before swelling. Perfusion of 50mM NMDA into guinea pig cochleae produces very similar ultrastructural damage to that seen in cochleae treated with 100μM kainate. However since the electrophysiological losses and surface structural damage produced by NMDA but not 100μM kainate can be attenuated by NOS inhibition and scavenging for superoxide, it seems likely that this similar ultrastructural damage is produced by different routes of toxicity.

11.3.2. Attenuation of excitotoxicity mediated by quisqualate and kainate by pre-treatment of the cochlea.

At all concentrations used, pre-treatment of guinea pig cochleae with L-NAME, L-MA, SOD or DEF did not afford any protection on cochleae from the excitotoxic action of either kainate or quisqualate as judged by electrophysiological and morphological criteria. Electrophysiological data alone cannot exclude the possibility that excitotoxicity had been reduced, because even if post-

excitatory depression of type I afferents (excitotoxicity) were attenuated/blocked, CAP loss could still occur if excitation of the type I afferents continued. Nevertheless, morphological examination revealed that there was no reduction in the degree of afferent nerve ending swelling, therefore it is unlikely that excitotoxicity was attenuated.

11.3.3. Comparisons with Previous Data

11.3.3.1. Involvement of NO in Excitotoxicity mediated by non-NMDA receptors

The results presented here imply that production of NO by NOS is not involved in the acute excitotoxicity produced by kainate and quisqualate. There are no comparative data concerning the role of NO in the excitotoxic action of non-NMDA receptors in the cochlea. There is some evidence that inhibition of NOS is neuroprotective against the action of kainate in retinal cultures (Dutrait *et al.* 1995). However, selective inhibition of neuronal NOS was unable to provide protection against striatal lesions produced by kainate and AMPA in vivo (Schulz *et al.* 1995b). In addition, cortical cultures from mice deficient in neuronal NOS were not resistant to kainate and quisqualate toxicity despite being resistant to NMDA toxicity (Dawson, Kizushi, Huang *et al.* 1996). The inconsistency of these data could be due to the use of different NOS inhibitors. However, since all inhibitors were capable of inhibiting neuronal NOS which is the predominant isoform of NOS in neuronal cells, this is unlikely. Therefore the current evidence suggests that non-NMDA receptors may mediate some toxicity through production of NO by NOS in some but not all neuronal cells. The ability of neuronal cells to produce NO in response to non-NMDA receptor activation may be related to the subunit composition of the receptors. Neuronal NOS (and endothelial NOS) is activated by an increase in the concentration of intracellular Ca^{++}. Activation of non-NMDA receptors can evoke an increase in intracellular Ca^{++} by voltage dependent Ca^{++} channels. In addition, certain subunits of the non-NMDA receptors can confer the ability to passage Ca^{++}. Therefore, cell types containing non-NMDA receptors which are permeable to Ca^{++} may produce greater increases in intracellular Ca^{++}, and therefore greater

production of NO by NOS. Alternatively, some neuronal cells may produce NO by other routes, for example, AMPA and metabotropic (type AP-3) glutamate receptors in the rat cerebellum produce NO via a NOS independent pathway (Yamada and Nabeshima 1997). In conclusion, several types of non-NMDA receptors may be able to produce NO by various different mechanisms. Therefore, although non-NMDA receptors do not appear to mediate excitotoxicity in the cochlea by NOS, an involvement of NOS independent production of NO has yet to be investigated.

11.3.3.2. Involvement of Superoxide and Hydroxyl radicals in Excitotoxicity mediated by non-NMDA receptors

The results presented here imply that neither superoxide nor hydroxyl radical are involved in the acute excitotoxicity produced by kainate and quisqualate. There are no comparative data concerning the involvement of ROSs in the excitotoxicity mediated by non-NMDA receptors in the cochlea. Ototoxicity following acoustic trauma and ischaemia/reperfusion is believed to be mediated predominantly by non-NMDA activation (Pujol *et al.* 1992; Pujol *et al.* 1993), and can be attenuated by treatment with SOD or allopurinol (an inhibitor of xanthinine oxidase which is a cellular source of superoxide) (Seidman, Shivapuja and Quirk 1993; Seidman, Quirk, Nuttal *et al.* 1991). However, morphological examinations were not carried out, and ischaemia/reperfusion and acoustic trauma involve additional mechanisms of ototoxicity besides those mediated by non-NMDA receptors. Therefore the protective effects of SOD and allopurinol may not have involved attenuation of toxicity which was mediated by non-NMDA receptors. The role of ROSs in non-NMDA mediated excitotoxicity is more clear in non cochlear tissue. A neuroprotective action of superoxide and hydroxyl radical scavengers or inhibitors of OH- production has been reported against kainate mediated excitotoxicity mediated in cortical, cerebellar and retinal cell cultures (Patel, Day, Crapo *et al.* 1996; Dugan, Gabrielson, Lin *et al.* 1996; Giusti, Gusella, Lipartiti *et al.* 1995; Dykens, Stern and Trenkner 1987; Dutrait *et al.* 1995). Until recently there was no evidence

of a similar protection against the excitotoxicity mediated by the AMPA receptor. However, Schulz *et al.* (1995a) reported that pre-treatment with a free radical spin trap (N-tert-butyl-alpha(2-sulfophenyl)-nitrone) significantly attenuated excitotoxic striatal lesions produced by kainate and AMPA *in vivo*. Therefore ROSs appear to be involved in mediating neurotoxicity produced by kainate and AMPA receptors in the CNS. Since both *in vivo* and *in vitro* neuroprotection conferred by antioxidants in the CNS often required a minimum of two treatments (one before and one after the excitotoxic event) (Schulz *et al.* 1995a; Giusti *et al.* 1995), single treatments given to the cochlea may not have been sufficient to provide protection. In addition, the in vivo protection in the CNS was determined 1 week post excitotoxicity whereas the experiments described here were acute (excitotoxicity was assessed 30 min post perfusion of quisqualate or kainate). Therefore the data presented here do not exclude an involvement of superoxide or hydroxyl radical in the excitotoxicity produced by kainate and quisqualate.

During this study, an unexpected finding was that scavenging for SOD and DEF appeared to have worsened the excitotoxicity produced by 100μM kainate in the cochlea. SOD and DEF appeared to enhance the ability of kainate to produce swelling of the type I afferent dendrites and CAP losses. Since ROSs have only been associated with toxicity and not protection in the cochlea, and SOD and DEF do not appear to produce ototoxicity on their own, this finding is difficult to interpret. *If* the scavengers selectively entered afferent neurons, they may have altered the osmotic gradient between the afferent neurone synapse and the extracellular space, which would produce greater swelling of the neurons. Alternatively, *if* superoxide and/or hydroxyl anions could antagonise kainate receptors, scavenging for these radicals could have lead to greater activation of kainate receptors by removal of these antagonists. However, this is pure speculation because there is no evidence to support either theory. An alternative explanation is that the difference in the responses to 100μM kainate between pre-treated and non pre-treated cochleae was simply due to inter-animal variation. According to Jenison *et al.* (1985) a 100μM dose of kainate is on the steep

slope of the dose response curve, therefore slight alterations in the concentration of kainate in the cochlea would have lead to large changes in the CAP losses produced. Data from cochlear perfusions is particularly variable with agents which produce CAP losses of the magnitude that 100μM kainate produced. Therefore although it is statistically very unlikely, the differences may have been purely down to chance. Clearly, further experiments are necessary.

11.3.4. Summary and Future Studies

In summary, although the results presented here do not provide any evidence for a putative role of NO, superoxide and hydroxyl radicals in the genesis of excitotoxicity produced by kainate and quisqualate, they do not preclude the possibility. Future experiments should involve administration of ROSs scavengers and NOS inhibitors both before and after perfusion of non-NMDA agonists, and excitotoxicity should be assessed several hours or days after perfusion. This may require use of the round window technique rather than the cochlear perfusion method because the physiological condition of the cochlea tends to deteriorate after 2-3 hours if the cochlear wall is fenestrated. With respect to the possible role of NO, an important step would be to determine whether activation of non-NMDA receptors in the cochlea results in production of NO by assaying the cochlear fluids or cochlear tissue for the presence of nitrites, nitrates (products of NO breakdown) and L-arginine (precursor of NO).

Non-NMDA receptor activation can evoke cell death through two main routes: osmotic swelling and lysis following the passive influx of Cl^-, and/or increased intracellular Ca^{++} which can cause cell death by several routes (including production of NO and ROSs). The contribution of either route varies between cell type (Monaghan, Bridges and Cotman 1989). The ultrastructural damage produced by 100μM kainate implies that osmotic swelling is not the only type of excitotoxicity produced by non-NMDA receptors in the cochlea because there was mitochondrial and membrane damage despite no apparent swelling of the nerve ending itself. However, higher concentrations of non-NMDA agonists produce striking swelling of the afferent nerve endings which suggests that the predominant form of toxicity is mediated by osmotic swelling. *If* non-NMDA mediated excitotoxicity in the cochlea is produced predominantly by osmotic swelling and lysis, it would be unlikely that inhibition and scavenging of NOS and ROSs respectively could attenuate this excitotoxicity. This could be tested experimentally by adjusting the Ca^{++} and Cl^- concentrations in

the extracellular medium of cultured type I afferent neurons and the Cl^- concentration in the perilymph *in vivo*.

Another approach to reduce excitotoxicity in the cochlea would be to utilise neurotransmitters acting on the lateral efferents. There are at least 6 neurotransmitters at the lateral efferent synapse including Ach, GABA and dopamine (Pujol *et al.* 1995). Dopamine depresses the increased activity in type I afferents produced by glutamate, NMDA, kainate or AMPA (Oestreicher, Arnold, Ehrenberger *et al.* 1997). 1mM Piribedil (a dopamine D2/D3 agonist) has been shown to attenuate swelling of afferent neurons induced by ischaemia (Pujol *et al.* 1993) or acoustic trauma (d'Aldin, Puel, Leducq *et al.* 1995), and significantly attenuated the CAP threshold shifts produced by acoustic trauma. In addition, enkephalins inhibit presynaptic release of Ach in the cochlea. Therefore it is likely that dopamine (and possibly enkephalins) may be capable of attenuating the excitotoxicity produced by non-NMDA agonists by reducing the induced increase in activity in the type I afferents.

Since AMPA and kainate mediated excitotoxicity, and ototoxicity following mild ischaemia and mild acoustic trauma is reversible within 5-10 days (Puel, d'Aldin, Ruel *et al.* 1997; Zheng, Henderson, Hu *et al.* 1997), an alternative approach would be to investigate the factors which inhibit or enhance re-innervation following non-NMDA mediated excitotoxicity. Recent data suggests that NMDA and metabotropic receptors are upregulated in primary auditory neurons during re-innervation of the inner hair cells following AMPA induced excitotoxicity (Puel, Saffiedine, d'Aldin *et al.* 1995), and NMDA antagonists lengthen the repair time (Puel *et al.* 1997). Yet, if given after an excitotoxic insult NMDA antagonists can reduce excitotoxicity (presumably by inhibiting the action of excitotoxicity mediated glutamate release). Therefore it is essential to understand these processes in order to enhance repair as well as reduce excitotoxicity.

11.3.5. Use of the Cochlear Perfusion Model in studies involving possible protection against the action of ototoxic agents.

The results presented here demonstrate that in order to determine whether pre-treatment of the cochlea with appropriate substances provides any protection against the action of ototoxic agents, it is essential to establish a concentration of the ototoxic agent that produces a reliable level of ototoxicity, and to pre-treat the cochlea with agents that are not themselves ototoxic. If the substance used to pre-treat the cochlea is itself ototoxic, the significance of further losses produced by other agents becomes unclear. If the ototoxic agent is too potent (CAP loss>60dB), the hearing loss is difficult to measure because the threshold CAP amplitude cannot be produced by maximum intensity stimulation, and is therefore highly dependent on the initial threshold. In addition, a small degree of attenuation may not be detected. Equally, if the ototoxic agent is not potent enough (15-25dB CAP loss) the inter-animal variation in susceptibility to such ototoxic agents is greater, and cochlear damage is less detectable in terms of ultrastructural damage. For optimum results, the concentration of the ototoxic agent should be adjusted to produce CAP losses of around 30-50dB.

If pre-treatment substances are not ototoxic, and ototoxic agents produce CAP losses of around 30-50dB, the cochlear perfusion model is a suitable method to investigate the effect of pre-treating the cochlea on the ototoxicity produced by various agents. However, morphological examinations are an essential part of the investigations in order to verify whether the degree or type of ototoxicity has changed. An alternative method may be to administer potentially protective drugs systemically (or i.c.), particularly as this is the most likely route by which protective agents would be administered clinically.

11.4. Role of excitotoxicity and ototoxins in meningitis-induced deafness

The main lines of evidence for an **involvement of ototoxins in meningitis-induced deafness,** discussed in chapter one, were: i) cochlear damage/infection is always associated with hearing losses ii) the early onset of meningitis-induced deafness precludes the involvement of complications of the inflammatory response; iii) data from isogenic mutant pneumococci implies that pneumolysin is the major cause of hearing loss in experimental pneumococcal meningitis; and iv) there is a similarity between cochlear damage produced by purified pneumolysin and experimental pneumococcal meningitis. The data presented here provides further support for the role of ototoxins in meningitis-induced deafness. There was some evidence that ototoxins exist in the bacterial extracts of all three major meningogenic pathogens, and the relative ototoxicity of each extract (as judged by CAP losses) was proportional to both the relative concentration of "pneumolysin-like" proteins in each extract, and the incidence of hearing loss in clinical meningitis produced by each pathogen.

However, other lines of evidence do not support the view that ototoxins are the sole contributor to meningitis-induced deafness. First, inflammation undoubtedly contributes, probably by exacerbating rather than initiating deafness because there is some evidence that adjunctive anti-inflammatory treatment is effective in reducing experimental and clinical meningitis-induced deafness (reviewed in Townsend and Scheld 1996). Second, almost all of the direct evidence to indicate ototoxins in the genesis of meningitis-induced deafness comes from experimental meningitis induced by i.c. inoculation without antibiotic treatment. These experiments produce a far higher incidence of hearing loss than seen clinically. Although factors such as the size and structure of the cochlear aqueduct, the route of meningeal infection, and concentration of bacteria used to inoculate the CSF may be responsible for the greater incidence of hearing loss in experimental meningitis, omission of antibiotic treatment during experimental meningitis is the

most likely cause. Losses in the size of the CAP produced by S. pneumoniae type II perfused directly into the cochlea were significantly reduced by pre-treatment with cefotaxime or amoxycillin (Winter, Comis, Osborne *et al.* 1998). In addition, Bhatt *et al.* (1995) treated rabbits with antibiotics during experimental pneumococcal meningitis, and found that although all animals suffered initial hearing losses, several animals regained their hearing by 2 weeks. The reversible hearing losses produced by experimental pneumococcal meningitis suggest that either the hearing losses produced by pneumococcal ototoxin/s were reversible, or ototoxin/s 'like pneumolysin' were not responsible for the hearing losses in these animals.

In conclusion, although the ototoxin pneumolysin appears to be a major factor in the onset of meningitis-induced deafness in untreated experimental pneumococcal meningitis, definitive investigations are necessary to implicate ototoxins such as those suggested in this thesis as being responsible for hearing losses produced by pathogens such as *H. influenzae* and *N. meningitidis*. In addition more experiments involving antibiotic treatment and long term assessments of hearing (several days) are necessary to determine whether ototoxins are involved in transient and permanent meningitis-induced hearing losses produced in animals treated with antibiotics.

The main lines of evidence for an **involvement of excitotoxicity in meningitis-induced deafness**, discussed in Chapter one, were: i) there is an increase in the concentration of excitatory amino acids in the CSF during experimental and clinical meningitis: ii) high concentrations of excitatory amino acids are ototoxic; ii) glutamate antagonists are neuroprotective in experimental meningitis; and iv) ototoxicity produced by pneumolysin can be attenuated by antagonists of the NMDA receptor. There is now additional supportive evidence. The ultrastructural damage produced by bacterial extracts and live pneumococci (inoculated in the CSF or cochlea) often involves selective damage to the afferent neurons which is similar to that produced by 50mM NMDA (unpublished data from our laboratories) or a low concentration (100μM) of the non-

NMDA agonist kainate (this thesis). Moreover, swelling of the afferent neurones very similar to that produced by non-NMDA agonists (this thesis) has been seen following experimental pneumococcal meningitis (Winter, unpublished findings). There is also a similarity in the time course of recovery in reversible hearing losses following non-NMDA mediated excitotoxicity and clinical or experimental meningitis. CAP losses produced by the non-NMDA agonists AMPA and kainate recover within 5-10 days (Zheng *et al.* 1997; Puel *et al.* 1997), ABERs were seen to return to control levels between 5 days and 2 weeks after inoculation in experimental pneumococcal meningitis (Bhatt *et al.* 1991), and clinically, reversible hearing losses return to normal 18 h to 5 days after diagnosis (Richardson 1997).

However, other lines of evidence do not support excitotoxicity as the **sole** factor responsible for meningitis-induced deafness. *First,* OAEs are not affected by kainate (Zheng *et al.* 1996) but were affected in all children with transient or permanent meningitis-induced hearing losses (Richardson 1997). *Second,* the early onset of all meningitis-induced hearing losses precludes the involvement of ischaemia which is the most likely source of excess glutamate release. However, it must be pointed out that bacteria contain glutamic acid in peptidoglycan and cytoplasm, therefore bacterial sources of excitatory amino acids could be responsible for early excitotoxic damage in the cochlea.

In conclusion, despite a lack of *direct* evidence, it is conceivable that excitotoxicity in the cochlea during meningitis may be, responsible for, or contribute to, some (particularly transient) hearing losses. If this were the case, it is likely that both non-NMDA and NMDA receptors are involved.

11.5. Antibiotic Treatment

In experimental meningitis, the most important treatment in order to reduce the incidence and degree of hearing loss appears to be early antibiotic treatment because antibiotics significantly reduced the incidence and degree of labyrinthitis and hearing loss in experimental *H. influenzae*

type b and *S. pneumoniae* meningitis (Kaplan *et al.* 1989, Bhatt *et al.* 1995) and in cochlear perfusion of pneumococci (Winter, Comis, Osborne *et al.* 1998). In these studies, antibiotics were given early in the course of the infection, and so are likely to have reduced the cochlear damage by reducing the total amount of toxins released by hindering multiplication of bacteria. Clinically there have been several reports which indicate that there is a correlation between meningitis-induced hearing loss and length of time the patient was unwell before admission. This would imply that late antibiotic treatment is associated with a greater incidence of hearing loss. Other reports have not shown this, but this may be because of the difficulty in determining the exact onset of the infection clinically. Since the onset of meningitis-induced deafness occurs early in the course of meningitis, the way in which late antibiotic treatment could lead to a higher incidence of meningitis-induced deafness is most likely to have been by making existing hearing losses permanent rather than inducing them.

11.6. A proposal for the site of damage responsible for transient and permanent meningitis-induced hearing losses

The morphological and electrophysiological data from experimental meningitis, and cochlear perfusion of bacterial extracts/toxins suggests that the primary site of cochlear damage is often the nerve endings, whereas hair cell damage only occurs if cochlear damage progresses. Since auditory nerves, but not hair cells can regenerate, it is conceivable that nerve endings alone are the site of initial cochlear damage produced during meningitis which is responsible for either transient hearing losses, or the onset of permanent hearing losses, whereas hair cell damage is a later event responsible for making initial hearing losses permanent. However, OAEs are altered in patients with transient (as well as permanent) hearing losses. Nevertheless, a change in the OAEs could represent a change in the motile response of the hair cells in response to the nerve ending damage, and does not necessarily represent hair cell damage. Alternatively, initial cochlear damage could also involve cratering of supporting cells which would produce some loss of hair cell function by

the resultant drop in the endocochlear potential due to endolymph and perilymph mixing. This type of cochlear damage could be reversible by re-growth of the supporting cells.

Ototoxins are the most likely candidates for the initial nerve ending damage because they can be released early in the course of meningitis. Additional bacterial components such as glutamate (a constituent of some bacterial cell walls) and NO (which some bacterial can release) may also contribute to early cochlear damage. Later hair cell damage could involve several factors such as ototoxins, excitatory amino acids, NO, ROSs, other inflammatory mediators, and consequences of the inflammation such as ischaemia. It is most likely that a combination of these factors is responsible for the progression of cochlear damage to include hair cell damage, although those factors which can induce CM losses in cochlea are the most likely candidates (e.g. NO, ischaemia, certain ototoxins, and NMDA receptor activation).

The major determinant of whether cochlear damage progresses to include hair cell damage (i.e. hearing losses become permanent) is likely to be the timing of antibiotic treatment because if given early the antibiotics could prevent cochlear damage progressing beyond nerve ending damage by limiting the concentration of ototoxins and inflammatory mediators.

11.7. Meningitis-induced Deafness : Final Overview and Conclusions

In order to reduce the incidence and degree of meningitis-induced deafness, the most important treatment appears to be prompt antimicrobial therapy. However, adjunctive therapy may also be of use, particularly in patients which receive antibiotic treatment late in the course of the infection. The most likely effect of anti-inflammatory agents is to prevent hearing losses becoming permanent by eliminating the role which inflammation plays in cochlear damage. Additional agents which block later stages of toxicity such as NOS inhibitors and glutamate receptor antagonists

should also be considered because a considerable inflammatory response may have already been produced in patients which present late. Therefore, in terms of future research, I believe that apart from vaccination which is the most important preventative method, investigations into how initial hearing losses become permanent rather than how initial losses occur is most important because it is unlikely that the patient will present before some hearing losses occur, whereas the period of time in which reversible hearing losses become permanent is when treatment can be given.

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Appendix I

Composition of Solutions

Phophate Buffer (0.2M pH 7.2)

1.35g KH_2PO_4 in 50ml distilled H_2O

0.31g NaOH in 39ml distilled H_2O

Glutaradehyde Fixative (0.05M 2.5% Osmolarity = 350 mmolkg^{-1})

25ml 0.2M Phosphate Buffer

10ml 25% glutaraldehyde

1.71g sucrose

Distilled H_2O to make a final volume of 100ml

Osmium Tetroxide (0.05M 0.1% Osmolarity = 350 mmolkg^{-1})

2.5ml Phosphate Buffer

0.1g OsO_4

0.855g sucrose

Distilled H_2O to make a final volume of 10ml

TEM Embedding Resin

2.5ml Agar 100

3.3ml Araldite

4.2 ml Dodecenylsuccinic anhydride

0.15ml Dibutylphthalate

0.25ml 2,4,6-tri(dimethylaminomethyl) phenol

Artificial Perilymph (Osmolarity = 300 mmolkg^{-1})

8g NaCl (final concentration = 137mM)

0.37g KCl (final concentration = 5mM)

2ml 1mM $CaCl_2$ (final concentration = 137mM)

0.156 $NaH_2PO_4 \cdot 2H_2O$ (final concentration = 1mM)

0.202g $MgCl_2 \cdot 6H_2O$ (final concentration = 1mM)

1.01mg NaHCO3 (final concentration = 1mM)

1.982g Glucose (final concentration = 11mM)

Appendix II

Construction of the non-pneumolysin producing mutant of S. pneumoniae type III

Extracts from S. pneumoniae type III bateria were generously provided by Prof Tim Mitchell, Glasgow University (formerly University of Leicester).

The pneumolysin deficient S. pneumoniae type III from which the bacterial extract was derived was constructed by insertion-duplication mutagenesis (Berry, Paton and Hansman 1992). This process is briefly outlined below (Schematic representation in Figure AII).

A 690 fragment of the pneumolysin gene was excised from pJCP20 (a plasmid which carries the complete S. pneumoniae pneumolysin gene) and purified. This fragment of the pneumolysin gene was then ligated into the vector pVA891 (a plasmid which carries a streptococcal gene encoding erythromycin resistance), and transformed into E.coli DH1. The recombinant plasmid was then purified from its E.coli host. Since this plasmid could not be directly transformed with encapsulated S. pneumoniae, the recombinant was transformed with a non-encapsulated S. pneumoniae first (Rx1). Recombination between the plasmid inserts and the homologous regions of the Rx1 chromosonal DNA the chromosomal coding sequence for pneumolysin was interrupted. This was confirmed with Southern blot hybridisation. DNA from this organism was finally transformed into encapsulated S. pneumoniae type III and the failure to make a translated gene product confirmed with Western blot hybridisation and a haemolytic assay.

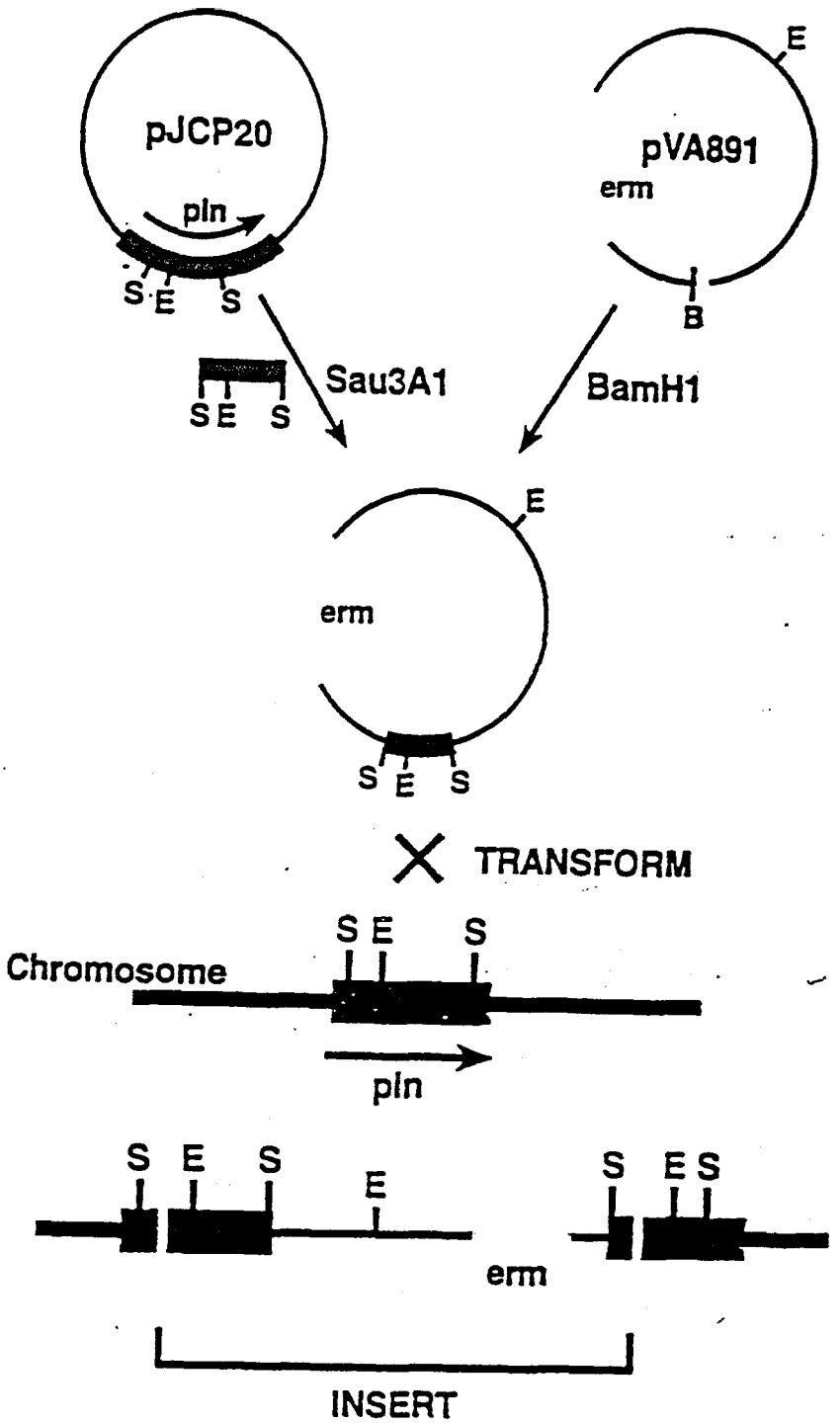

Figure AII. Schematic representation of the insertion-duplication mutagenesis of the S. pneumoniae pneumolysin gene (pln).

Erythromycin resistance marker (erm)

Restriction enzyme sites EcoR1, BamH1 and Sau3A1 (E, B and S respectively).