THE CLINICAL AND EXPERIMENTAL OTOTOXICITY OF AMINOGLYCOSIDES

By

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SUMMARY

A prospective investigation of the incidence of ototoxicity in 35 patients treated with gentamicin is reported. Auditory function was assessed by serial pure-tone audiometry and vestibular function by simple clinical tests. Studies on 24 normal subjects showed that portable audiometry on the ward was reliable. However, 3 of the 25 patients in the control group, none of whom received a known ototoxic drug, had hearing losses. This has important implications for the design and interpretation of this and similar studies. The reliability of the monothermal binaural hot caloric test as a screening procedure for detecting aminoglycoside vestibulotoxicity was assessed using normal subjects. Sustained reductions in response occurred in some subjects indicating that this test would be unsuitable.

Variation in binding of gentamicin to serum proteins is considered as a possible contributory factor to its toxicity. Irrespective of the method of study used, the binding of gentamicin to human serum proteins was found to be low and unlikely to be of clinical importance. Ribostamycin has been reported to be one of the least toxic aminoglycosides. The pharmacokinetics of this drug in the serum and perilymph of guinea-pigs were examined in relationship to its toxicity. Results on the effects of different fixatives on the ultrastructure of the organ of Corti are also reported.

KEY WORDS:

AMINOGLYCOSIDE
CLINICAL OTOTOXICITY
GENTAMICIN
RIBOSTAMYCIN PHARMACOKINETICS
PROTEIN BINDING

TO MY FAMILY

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SECTION 1

1.1 GENERAL INTRODUCTION

The ineffectiveness of penicillin in the treatment of infections due to Gram-negative organisms was the primary stimulus for the search for antimicrobial agents effective against such bacteria. Unlike the purely accidental discovery of penicillin, the development of streptomycin was a result of well-planned search begun in 1939 by Waksman and his colleagues. During their search for the desired antibiotic, many thousands of cultures, mostly actinomycetes, belonging to the genus Streptomyces, were isolated from soils, composts, manures, peat bogs and other natural substrates. These fungi and bacteria were grown in the laboratory and their metabolic products were tested for their antimicrobial activity against different bacteria, particular attention being paid to the mycobacteria. These workers isolated a large number of organisms which were found to possess considerable activity against Mycobacterium tuberculosis. Unfortunately, most of the antibiotics produced by these organisms proved to be unsuitable because their toxicity was too high in relation to their therapeutic activity. As a result of this work streptothricin and, later, streptomycin was discovered by Shatz, Bugie and Waksman in 1944. Great interest was aroused when it was found that this compound exhibited antibiotic activity against certain bacteria particularly the organism responsible for tuberculosis. For the first time in history of human tuberculosis, a drug had been found which could be used in its treatment and which pointed to the possibility of finally eradicating the 'white plague' off man.

A major disadvantage of streptomycin therapy emerged soon

after its initial clinical use. It became apparent that streptomycin could cause both disturbance of vestibular function and impairment of hearing (Hinshaw and Feldman, 1945). It was also found that after prolonged administration strains of streptomycin-resistant bacteria developed.

In recent years, although several new aminoglycosides have been discovered either from natural substrates or made semi-synthetically by molecular modifications of known structures, major problems of bacterial resistance and toxicity still remain. Thus there is still a need for research into the development of other agents in this class. Streptomycin, neomycin, framycetin, kanamycin, paromomycin, gentamicin and tobramycin are considered the primary generation of aminoglycosides, newer compounds of the second and third generation have now been found (Jackson, 1977). Those that have received considerable laboratory and clinical investigation are sisomicin, amikacin and netilmicin.

With the introduction of aminoglycosides, ototoxicity began to assume its rightful place as phenomenon of widespread clinical concern rather than a pharmacologic curiosity or an obscure side-effect. Ototoxicity may be defined as a tendency of certain therapeutic agents and other chemical substances to cause functional impairment and cellular degeneration of the tissues of the inner ear, and especially of the end-organs and neurons of the cochlear and vestibular divisions of the eight cranial nerve.

1.1.1 Streptomycin

Streptomycin was first isolated in 1944 from *Streptomyces griseus*. It is active against *Mycobacterium tuberculosis*, many Gram-negative bacilli and some staphylococci. Streptococci and pneumococci are relatively resistant, and anaerobic bacilli and

fungi almost completely insensitive. The original wide spectrum of activity of streptomycin against Gram-positive and Gram-negative bacteria has now narrowed greatly due to the emergence of resistant strains, and this has limited its usefulness. However, streptomycin is still a 'first line' drug in the treatment of tuberculosis. Martin (1970) has extensively reviewed its use in other infections. It is a drug of first choice in plague and tularaemia when it is commonly combined with tetracycline. Its use in acute brucellosis has now been superseded by doxycline or co-trimoxazole and its use in combination with penicillin in enterococcal infections has been superseded by penicillin combined with other aminoglycosides (e.g. gentamicin). Streptomycin with chloramphenicol has been advocated as the treatment of choice for Klebsiella pneumoniae pneumonia. Simon (1968) suggested that streptomycin should be reserved for tuberculosis, tularaemia and plague.

1.1.2 Neomycin

This antibiotic complex (neomycin A, B and C) is derived from a strain of Streptomyces fradiae and was isolated by Waksman and Lechevalier in 1949. Commercial preparations are a mixture which is largely neomycin B with a small amount of neomycin C. The spectrum of antibiotic activity of neomycin resembles that of streptomycin and includes various Gram-positive and Gram-negative bacteria as well as the tubercle bacillus.

Neomycin is absorbed only very slightly when administered orally, and is excreted unchanged in faeces. The oral route of administration is used to reduce the population of bacteria in the gastrointestinal tract in preparation for bowel surgery and has also been extensively used for supressing the intestinal flora in hepatic failure. The primary use of neomycin is for topical therapy of superficial infections of the eyes and the

skin, often in combination with other antibiotics, such as polymyxin and with adrenocorticosteroids. Neomycin is not used parenterally because it is very toxic.

1.1.3 Framycetin

In 1947, Decaris noticed a pink mould growing on a damp patch on the wall of his home in Paris. He cultivated the mould and identified it as a strain of Streptomyces lavendulae and found that culture filtrates were bactericidal for many species of Gram-positive and Gram-negative bacteria. These findings were not published until 1953 (Decaris, 1953). Further extraction and purification of the antibiotic were carried out in the research laboratories of Roussel. According to Rinehart, Argoudelis, Gross, Sohler and Schaffner (1960) framycetin is identical with neomycin B.

Administration of framycetin has been topical or oral. Skin infections and nasal carriers of staphylococci have been treated as with neomycin, and Escherichia coli enteritis has been successfully treated. Framycetin has been recommended for supression of intestinal flora prior to operation.

1.1.4 Kanamycin

This antibiotic was originally isolated in Japan in 1957 and is a product of Streptomyces kanamyceticus (Umezawa, Ueda, Maeda, Yagishita, Kondo, Okami, Utahara, Osato, Nitta and Takeuchi, 1957). Chemically it is similar to streptomycin and neomycin. It differs little from neomycin in anti-bacterial activity but may be slightly less active than neomycin against some strains of Proteus species, and is usually ineffective against most strains of Pseudomonas species (Welch, Wright, Weinstein and Staffa, 1958).

Kanamycin is a bactericidal antibiotic which may be effective when administered systemically for infections with susceptible

organisms involving the respiratory tract, urinary tract, bone and soft tissues or in septicaemias. It is usually reserved for serious infections in which less toxic antibiotics are not effective.

1.1.5 Paromomycin

This aminoglycoside was discovered in 1958 and was produced by a strain of Streptomyces rimosus originally recovered from a soil sample collected in Columbia. The antibiotic was first described by Haskell, French and Bartz (1959).

Clinical reports on the use of this antibiotic refer mainly to oral administration. The spectrum of activity of paromomycin is similar to that of neomycin (Coffey, Anderson, Fischer, Galbraith, Hillegas, Kohberger, Thompson, Weston and Ehrlich, 1959). In addition, the drug is an active amoebicidal agent against Entamoeba histolytica. It is used in the treatment of helminthiasis and amoebiasis.

Since paromomycin is not recommended for systemic use, it is presumably regarded to be too toxic for this purpose.

1.1.6 Gentamicin

Gentamicin was discovered in 1963 by Weinstein, Leudemann, Oden and Wagman from submerged fermentations of Micromonospora purpurea and Micromonospora echinospora isolated from loam and mud samples obtained in New York State. It is similar to aminoglycosides isolated from Streptomyces species. Gentamicin is a complex of three antibiotic substances, Gentamicin C1, $C1_a$ and C2 and has important advantages over previously described aminoglycosides, being exceptionally active against Pseudomonas aeruginosa (Barber and Waterworth, 1966). Gentamicin is active against most of the Gram-negative species, including Escherichia

coli, species of Klebsiella, Proteus, Pseudomonas, Shigella and Salmonella (Waitz and Weinstein, 1969). However, resistance of Pseudomonas aeruginosa has been recognized since the early 1970's (Shulman, Terry and Hough, 1971; Snelling, Ronald, Cates and Forsythe, 1971), and resistance rates of up to 20% in the U.S.A (Meyer, Halter, Lewis and White, 1976a), and up to 5% in the U.K (Draser, Farrell, Maskell and Williams, 1976) have been reported. Of equal importance in the U.S.A is the emergence of resistance in up to 50% of Serratia marcescens isolates (Meyer et al., 1976a) although this is not yet apparent in the U.K.

Gentamicin is inactive against anaerobic Gram-negative bacteria, and against Pseudomonas pseudomallei.

1.1.7 Tobramycin

The history of this antibiotic goes back to 1967, to the description by Stark, Hoehn and Knox (1967) of nebramycin, the product of a strain of Streptomyces tenebrarius. At least seven factors were identified in nebramycin (Thompson and Presti, 1968) of which that numbered six had the most desirable properties. This is now known as tobramycin. The outstanding property of this antibiotic is its activity against Pseudomonas aeruginosa exceeding that of gentamicin by about ten-fold, and retained against some gentamicin-resistant strains (Meyer, Young and Armstrong, 1971).

1.1.8 Sisomicin

Weinstein and colleagues (1970) reported the isolation of sisomicin (formerly referred to as rickamicin), which is produced by Micromonospora inyoensis. It is structurally related to gentamicin $C1_a$ and is active, but marginally less

effective, against the same spectrum of organisms as gentamicin and tobramycin (Draser, Farrell, Maskell and Williams, 1976). Sisomicin is active against various strains of bacteria, especially Rickettsia akari infections of mice.

1.1.9 Amikacin

Amikacin is a semi-synthetic aminoglycoside antibiotic with a broad antibacterial spectrum and is derived from kanamycin A (Bodey and Stewart, 1973).

It is resistant to many of the aminoglycoside inactivating enzymes which have been identified, including those responsible for gentamicin resistance. Unlike kanamycin, amikacin is effective against Pseudomonas aeruginosa and is active against many gentamicin resistant organisms (Reynolds, Hamilton-Miller and Brumfitt, 1974) including Serratia marcescens (Meyer et al., 1976a).

1.1.10 Netilmicin

Unlike sisomicin and amikacin, netilmicin has not yet reached the stage of extensive clinical trials.

Netilmicin is closely related to gentamicin $C1_a$, and like sisomicin is derived from Micromonospora inyoensis. Its activity lies between that of sisomicin and amikacin and is resistant to some, but not all, of the aminoglycoside inactivating enzymes (Kabins, Nathan and Cohen, 1976).

1.1.11 Ribostamycin

Shomura and colleagues (1970) isolated this antibiotic which is produced by Streptomyces ribosidificus and is a broad spectrum antibiotic. It is used in Spain and Japan but has not been used in the U.S.A and the U.K.

1.2 Use of aminoglycoside antibiotics at East Birmingham Hospital

East Birmingham Hospital is a District General Hospital of around 1000 beds. Most services are provided except for the maternity and gynaecology facilities. It is the only hospital in the Midlands which has an Infectious Diseases Department.

In 1978, a study was conducted by Talbot to determine the use of antibacterial agents at East Birmingham Hospital. His survey showed that streptomycin appeared to be confined to the treatment of tuberculosis and the use of kanamycin, tobramycin and amikacin was very limited. In six months only three prescriptions were recorded for tobramycin and one each for both kanamycin and amikacin. However, gentamicin was prescribed frequently throughout the hospital. There are no mandatory restrictions as to which antimicrobial agent can be used. Talbot's data indicated that aminoglycosides comprised ~ 5% of all the antibacterial agents used at East Birmingham Hospital.

1.3 Clinical studies

With the discovery of streptomycin came the finding that it could cause both disturbance of the vestibular and auditory functions (Hinshaw and Feldman, 1945). Other aminoglycosides subsequently introduced often demonstrated greater ototoxicity than streptomycin. As they came to be widely used for the treatment and prophylaxis of infections, the problem of drug ototoxicity became a matter of interest and concern both for the physician and for the otologist. Aminoglycoside ototoxicity is universally recognized and is regularly reproduced in animals. Despite the attention devoted to it, precise understanding of ototoxicity, its mechanisms and, more urgently, clinically useful means of avoiding it still elude us. This is not to imply that progress is not being made. For instance, certain contradictory statements in the early literature seem to have been reconciled and certain important generalizations about these ototoxic antibiotics can be made.

Whereas it was considered early on that aminoglycosides influenced hearing by damaging brainstem nuclei, it is now well established (Kohonen, 1965; Hawkins, 1970) that the primary site of damage is more peripheral. The clear-cut and reproducible destruction of the cochlear hair cells has led to the conclusion that these sensory elements are among the prime targets of these antibiotics.

Even before streptomycin was widely available, Hinshaw and Feldman (1945) published a report on the therapeutic potential of streptomycin. They described it as a drug of low toxicity, rarely producing any serious reactions in patients even on continuous therapy for several weeks. Unimportant

reactions such as pain at the site of repeated injections, moderate elevation of body temperature, histamine-like responses such as throbbing headache and flushing of the skin, mild malaise and aching muscles were reported. Of the 34 patients treated with streptomycin, four patients acquired an apparent sensitization to the preparation. They observed one case of transient deafness and three cases with vestibular disturbances. These symptoms were tentatively ascribed by Hinshaw and Feldman to a selective neurotoxic effect on the eight cranial nerve. However, they were not certain whether these symptoms were produced by streptomycin or some impurity since the first preparations available were not absolutely pure.

With the development of highly purified streptomycin preparations and the increasing experience on its use, the incidence of the inherent toxicity of the drug was reduced. Some of the early histamine-like manifestations reported were undoubtedly due to impurities in the first streptomycin preparations but the 'neurotoxic' effects reported were characteristic of many if not all streptomycin preparations. Since that report in 1945, numerous clinical studies have been published which confirmed the selective toxicity on the inner ear of not only streptomycin but of all the subsequent aminoglycosides discovered.

Clinical studies have revealed loss of vestibular and auditory function as well as nephrotoxicity attributable to all the aminoglycosides but the degree of damage differs (Tisch, Huftalen and Dickison, 1958; Lerner, Seligsohn and Motz, 1977; Smith, Baughman, Edwards, Rogers and Leitman, 1977). Auditory damage, manifested by varying degrees of sensorineural hearing

loss, especially for high frequencies (>4000 Hz), is characteristic of kanamycin, amikacin, neomycin and paromomycin. Vestibular impairment, resulting in disequilibrium, nystagmus, nausea, vomiting and vertigo, is more commonly associated with gentamicin, tobramycin and streptomycin. However, these distinctions are not absolute and any of the drugs can produce either or both forms of ototoxicity.

In animal experiments, including histopathological electron microscopic studies on human temporal bones, the toxic effect of aminoglycosides on the outer hair cells in the basal turn of the organ of Corti have been demonstrated (Benitez, Schucknecht and Bradenburg, 1962; Jorgensen and Schmidt, 1962; Lundquist and Wersäll, 1967; McGee, Webster and Williams, 1969; Igarashi, Lundquist, Alford and Miyata, 1971). The destruction then proceeds towards the apex (Farkashidy, Black and Briant, 1963). The initial manifestation may be tinnitus, although this condition is not always present, as was shown by Finegold's studies with kanamycin (Finegold, Winfield, Aronsohn, Hewitt and Guze, 1958; Finegold, 1966). Decreased hearing acuity may not be noticed by patients as the first changes shown by audiometry appear in the high frequencies. It may be noticeable only when the conversational frequency range is affected. Therefore, the 'tuning-fork test' in which the patient has to hear for the vibrations, will not always detect early damage (Arbit, 1979). Furthermore, if tinnitus is present it may interfere with the test.

Ototoxicity encountered with these drugs may be progressive with either bilateral or unilateral involvement (Dayal, Whitehead and Smith, 1975) though in certain circumstances it may be

reversible, sudden, delayed and severe continuing even after withdrawal of the offending medication (Finegold et al., 1958; Jackson and Arcieri, 1971; Tjernström, Banck, Belfrage, Juhlin, Nordström and Toremalm, 1973; Winkel, Hansen, Kaaber and Rozarth, 1978).

Because aminoglycosides are excreted entirely by glomerular filtration, renal impairment has been shown to have a close association with ototoxicity (Jackson and Arcieri, 1971). Other factors (Jackson, 1977) which have also been implicated are the prior or co-administration of other ototoxic antibiotics or diuretics (e.g. frusemide and ethacrynic acid).

It still remains uncertain whether a particular high (peak) serum concentration is a factor in the ototoxicity or whether it is rather the trough concentration which is decisive (Banck, Belfrage, Juhlin, Nordström, Tjernström and Toremalm, 1973; Nordström, Banck, Belfrage, Juhlin, Tjernström and Toremalm, 1973; Hewitt, 1974). In some cases high peak and trough levels have also been shown to produce no incidence of hearing loss (Dobbs and Mawer, 1976; Panwalker, Malow, Zimelis and Jackson, 1978). Panwalker et al. (1978) showed that two patients who developed extraordinarily high serum levels of netilmicin for periods of two to three weeks (peak levels as high as 36 µg/ml - range 15 to 36 µg/ml) and trough levels as high as 24 µg/ml (range 10 to 24 µg/ml) did not demonstrate any ototoxicity discernible by audiometry. These high levels were deemed necessary for cure in an immunosuppressed patient with a moderately resistant organism. On the other hand, one patient was found to have a reversible unilateral hearing loss with usual therapeutic levels. This study indicated that ototoxicity occurring in

the course of treatment is not entirely predictable and not related only to the plasma concentrations of the drug.

Immediate and reversible depression of cochlear activity in humans without clinical evidence of impairment, has been shown to follow single intravenous doses of tobramycin (Wilson and Ramsden, 1977). Electrocochleography was performed on three patients to monitor the effects of the loading dose of intravenous administration of tobramycin. Immediate reduction in cochlear output was observed in two patients when peak serum levels exceeded 8 - 10 µg/ml. In the third patient lesser changes occurred even though the serum levels were in the 'safe' range (less than 8 µg/ml). Such a rapid onset of electrophysiological changes is consistent with a temporary block in metabolic activity (inhibition of energy producing processes or blocking of transport of cations across cell membranes). In two of the three patients, peak serum levels greater than 8 µg/ml were sustained for 30 min or longer, but normal electrical activity was restored as soon as serum levels fell. No auditory or vestibular symptoms were experienced during or after treatment.

There is considerable disagreement as to the incidence of ototoxicity caused by the aminoglycosides. In part, this may be because of differences in the accuracy of adjustment of dosages in earlier studies, and to variable attention to the detection of sub-clinical damage by means of electronystagmography (ENG) and audiometry.

Jackson and Arcieri (1971) surveyed the case summaries of 1484 cases treated with gentamicin throughout the United States between 1966 and 1969. Among these there were 42 courses

accompanied by symptoms and/or signs that suggested vestibular or auditory dysfunction. This suggested the incidence of ototoxicity associated with gentamicin treatment to be 2.8%. On critical evaluation, about one-third of the cases reported were eliminated as doubtful and the true incidence of gentamicin-induced ototoxicity for the four year period was found to be about 2%.

Jackson and Arcieri's study further showed that in two-thirds of the patients with ototoxic side effects, dysfunction was limited to the vestibular system; only one-third of the patients had a change in hearing ability. Among the latter, vestibular symptoms were also present in one-half of the group. Symptoms of ototoxicity generally appeared between the first and second weeks of treatment, but were observed as early as the third day in a few instances. However, there was one patient in which symptoms were unrecognized until the ninetieth day of treatment.

Effects were transient in more than one-half of the patients with labyrinthine dysfunction. In others there was no evidence of recovery of function. Hearing loss was mild and restricted to high frequencies in approximately one-half of the patients who developed auditory changes.

Finally, Jackson and Arcieri pointed out that although it was helpful to know the frequency of serious reactions to a drug under general conditions of use, on the other hand, figures of overall incidence are not meaningful unless the importance of variations among patients and different features of the therapeutic regimen are known. In their study, 14 variables were analyzed in matched sub-groups. They concluded that of

these 14 variables, the dominant factor by far was the functional status of the kidney. Two-thirds of all patients with recognized ototoxicity had renal impairment. It was also found that with or without renal dysfunction, the daily dose of gentamicin according to the weight of the patient was the most important feature of the dosage regimen. Thus, toxicity was said to be the result of high concentrations of the drug in serum but the critical level for the production of ototoxicity was unknown.

Smaller studies by other workers substantiate the fact that renal insufficiency is of some significance in predisposing to ototoxicity. In a retrospective study (Banck et al., 1973) patients were chosen in whom vestibular damage was most likely i.e. patients treated for a long time with gentamicin or with a large total dose, or with renal insufficiency, or previously treated with ototoxic antibiotics. Some patients with symptoms of dizziness during and shortly after gentamicin treatment were also included. This pilot study was preparatory to a planned prospective investigation of potential risk factors associated with ototoxicity.

28 of 35 patients examined revealed nothing after electronystagmography (ENG) with caloric testing although five patients had vertigo during or immediately after treatment. Seven patients showed ENG abnormalities and all had severe, bilateral vestibular dysfunction. Hearing was also impaired in these seven patients, but only one noticed such impairment developing during treatment. Although this study did not justify any conclusions, it was pointed out by the authors that the risk of side-effects of gentamicin therapy appeared to be increased in patients treated previously with ototoxic drugs

and in patients with renal insufficiency as judged from the serum creatinine level. Individual tolerance to gentamicin was found to vary widely making it difficult to assess the risk of side-effects in any given case. In this investigation critical serum levels did not appear to be the important risk factor in any of the cases of ototoxicity.

In the same year results from the prospective study of gentamicin were reported (Nordström et al., 1973). A small group of patients (34) receiving gentamicin were investigated. In three patients the relationship between pathological findings and the administration of gentamicin were regarded as certain, which gave the incidence of vestibular ototoxicity of about 10%. These patients, all of whom exhibited vestibular symptoms during or shortly after treatment, were also reported to have severely abnormal ENG's with caloric stimulation. None of these patients had received any ototoxic antibiotics previously. It was found that there was a significant correlation between vestibular dysfunction and the serum creatinine levels thus indicating that impaired renal function increased the risk of vestibular ototoxicity due to gentamicin. The discrepancy between these findings on the incidence of ototoxicity and Jackson and Arcieri's (1971) study were explained by Nordström et al. to be partly due to differences in design and material (i.e. patient characteristics) and by the different ways used to express the frequency in the two investigations. It was also stated that in their study a higher percentage of patients had impaired renal function. Furthermore, they found that the relative age of patients was higher and also 50% of those with pathological ENG's had received two series of gentamicin

treatment which may have contributed to the differences in the incidence of ototoxicity reported, although the effect of age was shown to be insignificant statistically.

Tobramycin was associated with ototoxicity in less than 1% of Neu's series (Neu and Bendush, 1976), while Fee, Vierra and Lathrop (1978) demonstrated it in approximately 26% of their cases.

Introduction of new aminoglycosides has provoked much interest in the relative toxicity of these drugs. It is unfortunate that lack of commonly accepted criteria for defining ototoxicity makes valid comparisons difficult in human studies. Very narrow limits are adopted by some workers who consider that a hearing loss as small as 10 decibels (dB) at any one frequency using air conduction audiometry to represent changes related to therapy (Black, Lau, Weinstein, Young and Hewitt, 1976; Smith, Baughman, Edwards, Rogers and Leitman, 1977; Winkel et al., 1978). In physiological terms both the air and bone conduction tests should demonstrate a hearing loss if a change has occurred with these antibiotics. This hearing loss should normally be greater than 15dB when the final audiogram is compared with the initial or baseline audiogram.

To be effective, an antibacterial agent must intervene with an essential metabolic process in an interfering pathogen without having a delerious effect on the host. No antibacterial agent available fulfils these requirements completely. Some have toxic effects on the host. Bacterial resistance and the potential for toxicity are the major limiting factors in the effective use of the present aminoglycoside antibiotics. The ratio between therapeutic and toxic serum levels is narrow.

Clearly, it is logical to look at the area of side-effects and resistance and inquire whether we need these new aminoglycosides or if the continual research that is underway with compounds of this type will yield agents of true clinical superiority to the first generation aminoglycosides which have been in use for over three decades. On the basis of available data, it may be safe to say that these new aminoglycosides may not offer advantages over the previous aminoglycosides (e.g. gentamicin). It is possible that over the next few years, aminoglycosides may disappear from the medical armamentarium and be replaced by other antibiotics with a wide spectrum of activity. β-lactam antibiotics which from many points of view have possibly reached perfection. Their bacterial spectrum is very similar to many aminoglycosides and they seem to demonstrate no toxicity.

SECTION 2

RESPONSE DECLINE WITH REPEATED CALORIC TESTS OF VESTIBULAR FUNCTION IN NORMAL SUBJECTS

2.1 Introduction

Until the introduction of streptomycin, vestibular ototoxicity in the sense of a specific injurious action of a drug on the sense organs of the labyrinth had seldom been observed in the laboratory and was virtually unknown in the clinic. With increasing experience of the use of streptomycin over prolonged periods in the treatment of tuberculosis it became clear that this drug could cause damage to the eight nerve. Brown and Hinshaw (1946) reported on 23 tuberculous patients treated in the initial clinical trial of streptomycin who developed symptoms suggesting damage to the eighth nerve. The most common of these was described as 'a peculiar disturbance of equilibrium', without true vertigo but characterized by dizziness or light-headedness. At first this sensation was experienced only upon turning the head. With continuing treatment it became more or less constant, and could be controlled only by keeping the eyes closed. Several patients reported nausea. Those who were ambulatory became ataxic, at least to the extent of not being able to walk in a straight line. Most of them did not require assistance in walking, but they kept close to a wall for support when necessary. Vestibular tests indicated decreased sensitivity to stimulation, and in some nystagmus was entirely absent, whether the stimulus was caloric or rotational.

For some time after its discovery, streptomycin appeared to be more or less unique in its attack on the vestibular end organs. The potent ototoxic action of neomycin and kanamycin

is largely confined to the cochlea (Hawkins and Lurie, 1953). With gentamicin (Lundquist and Wersäll, 1967; Igarashi, Lundquist, Alford and Myata, 1971), cochlear injury is not uncommon but vestibular toxicity appears to be dominant, at least in man (Jackson and Arcieri, 1971). There is considerable disagreement concerning the incidence of ototoxicity caused by aminoglycosides. This is partly due to the methods employed and also due to the major differences in the populations studied.

Jackson and Arcieri (1971) reviewed the case histories of 1,484 patients treated with gentamicin throughout the U.S.A between 1966 and 1969. There were 42 reports of gentamicin treatment being associated with symptoms and/or signs which suggested vestibular or auditory dysfunction giving an incidence of 2.8%. On critical analysis, however, about one-third of the toxic effects reported were probably unrelated to gentamicin, making the true incidence for the four year period approximately 2%. Their study further showed that in approximately two-thirds of the patients with ototoxic side-effects, dysfunction was limited to the vestibular system.

Nordström et al. (1973) prospectively investigated a much smaller group of patients (34) receiving gentamicin therapy. Vestibular toxicity which was unequivocally attributable to gentamicin occurred in three patients (10%). These patients, all of whom exhibited symptoms of vestibular function, were reported to have 'severely abnormal ENG's' with caloric stimulation. Nordström et al. (1973) considered that the discrepancy between their findings and those of Jackson and Arcieri (1971) could be partly explained by differences in design and material (i.e. patient characteristics in the two investigations).

An accurate incidence of aminoglycoside induced vestibular ototoxicity can be determined only by systematic monitoring of the vestibular function of each patient receiving the drug. As it is seldom possible to transport sick patients from the ward to the Ear, Nose and Throat (E.N.T) Department to carry out these tests, a simple monitoring test would have to be applied on the ward using portable equipment.

The caloric test, involving thermal stimulation of the labyrinth, is the most commonly employed method of assessment of vestibular function. Caloric stimulation is an unphysiological procedure and does not reflect the normal response of the vestibular sense organ in any way. Nevertheless, it is the only way to investigate the function of each labyrinth separately; normal physiological stimuli always stimulates both the vestibular organs at the same time. In recent years, there has been an effort to improve the diagnostic usefulness of the test by carefully controlling irrigation fluid temperature and recording eye movements for detailed analysis.

During the caloric test, the change in temperature caused by syringing the ear with water at a temperature differing not too much from the body temperature is transmitted to the structures in the temporal bone towards the semi-circular canals. The first canal reached is the lateral canal in its most lateral part. Not only is the temperature of the bony wall of this canal changed but the surrounding endolymph is also influenced, for its specific gravity is lowered by heating and raised by cooling (Jongkees, 1973). In this way a flow of endolymph is produced that is dependent on the position of the canal. The maximal effect of the caloric test is produced

in the lateral semi-circular canal when it is in a vertical position. Thus, the strongest vestibular response is elicited when the patient is examined lying in the supine position with his head elevated at an angle of 30^{o} above the horizontal. Intralabyrinthine temperature changes resulting from aural irrigations are influenced by such variables as the efficiency of heat transfer between the irrigation fluid and the temporal bone, pre-existing thermal conditions within the temporal bone, and the rate of heat exchange with surrounding structures with blood (Schmaltz, 1932; Cawthorne and Cobb, 1954). The standard bithermal caloric test, which was first described by Fitzgerald and Hallpike (1942), consists of successive irrigation of each ear for 40 seconds with hot ($44^{o}C$) and cold ($30^{o}C$) water. The induced nystagmus may be recorded by the technique termed electronystagmography (ENG). The changes in corneo-retinal potential which occur with eye movement are amplified and recorded on an oscillograph. The recorded nystagmus is seen as a saw-tooth wave. As the eyes rotate in one direction the electrodes record a steady voltage increase. When the maximum angular rotation is reached, the direction of rotation is reversed. The eyes then return to the neutral position at a faster rate, recording a rapid drop in voltage. The saw-tooth tracing thus indicates the slow and fast components clearly (Spector, 1968). This nystagmus has various properties that can be used as parameters for its evaluation. Some of the most important ones are:

Latency.

Latency is dependent on the structure of the temporal bone. A sclerotic temporal bone allows far more rapid

conduction of temperature than a normally pneumatized bone. Cerumen in the external auditory canal can easily lengthen the latent period (Jongkees, 1973).

Duration.

Duration is an easy parameter to measure but it is not the best parameter of vestibular responsivity. Jongkees (1973) showed a poor correlation between the physical stimulus applied and the duration of the nystagmus reaction. He found a certain increase of the duration when a difference in temperature between the water used for irrigation and the temperature of the test person increased within a difference less than $7^{o}C$. When larger differences were used, there was no corresponding increase of duration.

Number and frequency of nystagmus beats.

The total number of beats and their frequency can be counted easily and have been found to be good indicators of the induced response. Frequency has been proven to be as reliable a measure as the speed of the slow phase (Torok, 1973). However, frequency of the caloric nystagmus can vary greatly especially when the mental activity of the patient changes. The most stable results are obtained by recording the nystagmus in the dark from a mentally active patient (by presenting the patient with a mathematical problem).

Speed of the slow phase.

The maximum speed of the slow phase has been found to correlate better with stimulus intensity (Henriksson, 1956). It varies in direct proportion to the temperature of the stimulation used. Subsequently, it was expressed that the slow phase of the nystagmus is a direct function of cupular

deviation (Henriksson, 1955b).

Total amplitude.

The total amplitude i.e. the sum of the amplitudes of all the nystagmic beats, represents the total deviation of the eye if it had not been interrupted by the quick phase. The total amplitude can only be determined when the nystagmus has been recorded accurately. It has been reported by some investigators to be very precise parameter (Jongkees, 1973), but it is very time consuming to measure and calculate, even from excellent recordings. However, it does correspond closely to the speed of the slow phase (Jongkees, 1973).

The use of the hot as well as cold stimuli allows nystagmus direction differences to be distinguished from vestibular responsiveness differences. However, the combination of the four irrigations together with the unavoidable waiting time between each irrigation, makes it a long lasting procedure. Consequently, there have been many attempts to shorten the test procedure and a survey of the literature points to the prevalence of the hot water tests (Bernstein, 1965; Hart, 1965; Hinchcliffe, 1967; Barber, Wright and Demanuelle, 1971) as an alternative to the bithermal caloric test. The hot caloric test (Hinchcliffe, 1967) is generally considered to be more reliable than the cold test which can give spurious results due to the spontaneous nystagmus induced by stimulation of the external meatus. The direction of this spontaneous nystagmus is always away from the ear being stimulated i.e. the same direction as the nystagmus induced by cold stimulation of the lateral semi-circular canal. Furthermore, use of the hot stimulus permits a check on the 'flush sign', (Coles, 1972) which is

seen as a reddening down the handle of the malleus after a hot irrigation that reaches the tympanic membrane.

Dayal, Farkashidy and Kuzin (1973) recommended the use of serial hot caloric test to monitor drug-induced vestibular ototoxicity. However, they claimed that although the hot caloric test was inadequate for detecting the excitability difference between the labyrinths, it might be useful in serial monitoring of bilateral vestibular ototoxicity caused by drugs.

In this study, it was proposed to adopt the hot caloric test as a serial monitoring procedure for potential aminoglycoside vestibular ototoxicity. Before applying it on sick patients, the test was performed on normal subjects to assess both between-subject and within-subject, between-test variation. Ideally, the test should be conducted in total darkness with eyes open. Since it would be impossible to achieve total darkness on the ward, it was decided to use Frenzel's glasses in a semi-dark room to abolish fixation. The glasses are illuminated goggles with plus twenty diopter lenses that inhibit fixation. The lenses magnify and illuminate the eyes thus, making it easier to see the nystagmic response by direct observation (Nelson, 1969). Baloh, Sills, Solingen and Honrubia (1977) have shown that while the nystagmus response is reduced using Frenzel's glasses compared to testing with eyes open in darkness, the consistency of the response on retesting is equal to that with eyes open in the dark and better than that with eyes closed or open in light.

Various nystagmic-response measurements have been proposed by different authors. In this study the maximum velocity of the slow phase and the maximum frequency were used

to determine the caloric-response intensity. The maximum velocity of the slow phase (Jongkees and Philipszoon, 1964) and the maximum frequency (Torok, 1969) have been reported to be better measures of the caloric-response magnitude as they correlate better with stimulus intensity.

2.2 Methods and Materials

2.2.1 Subjects

25 normal volunteer subjects of both sexes (15 male, 10 female) between the ages of 20 and 60 years were used (7 each in decades 20 - 30 and 30 - 40; 6 in 40 - 50 decade and 5 in 50 - 60 decade). Their auditory thresholds were within normal limits and they had no medical history of neuro-otological disease. The subjects were examined for any spontaneous, positional or paroxysmal positioning nystagmus. Subjects were not regularly taking central nervous system depressants and were told to abstain from alcohol for 24 h prior to the test day; their co-operation in this was confirmed just before the test. Prior to testing, the ears were examined to ensure intact tympanic membranes.

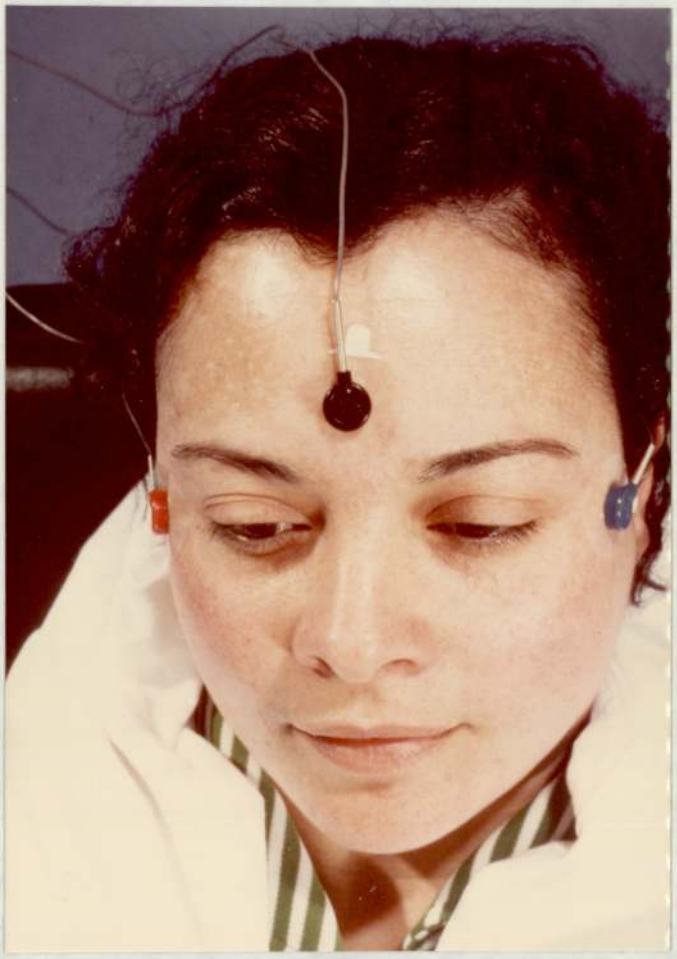
2.2.2 Preparation of the subject

The horizontal nystagmus responses were detected by three silver-silver chloride disc electrodes (Amplivox) and were recorded by ENG on a George Washington 400MD series pen oscillograph. Electrode cream was used to ensure that a good electrical contact was maintained between the skin and the electrode. Two of the electrodes were attached at the outer canthi of the eyes by means of (Amplivox) self-adhesive discs. The subject was earthed by means of the third electrode which was attached on the forehead (Fig. 1). The skin, where the electrodes were attached, had been previously cleaned thoroughly with alcohol to decrease resistance.

2.2.3 Calibration

Eye-movements were calibrated by having each subject move his eyes through a 12.5 degree arc prior to and following

Fig. 1 Arrangement of the electrodes for electronystagmographic recording. The two active electrodes were attached to the outer canthi. The subject was earthed by means of the third electrode which was attached on the forehead.



the test procedure ($12.5^{o} \equiv 12.5$ mm). An upward deviation of the pen represented eye movement to the right and a downward deviation represented eye movement to the left. The subject was asked to fix on a point about 1 metre in distance and was then asked to rapidly shift his glance to another point 12.5^{o} to one side of the first. The two points were in the subject's direct line of vision when lying in the caloric test position. When the subject performed these calibration movements the amplification level was adjusted so that the pen deflection corresponding to an eye movement of 12.5^{o} was 12.5 mm $\pm$ 1 mm. Quick re-calibrations were performed during the pause between successive caloric irrigations as, in prolonged tests, the electrode/skin resistance may vary slightly probably due to autonomic phenomena (Aschan, Bergstedt and Stahle, 1956) and the corneo-retinal potential is also liable to change due to alterations in amount of illumination during the course of the caloric tests.

2.2.4 The test procedure

The subjects were placed in the supine position with the head elevated at an angle of 30^{o} above the horizontal, thus bringing the lateral semi-circular canal into a vertical plane. In this plane, the lateral canal is maximally responsive to caloric stimulation.

Immediately after calibration Frenzel glasses were placed over the subject's eyes and the main lights were extinguished. The only remaining light was a 60 watt lamp placed to illuminate the ear to be irrigated. This light was extinguished at the end of the irrigation leaving the room in near darkness. Each ear was irrigated with water at $44^{o}C$ for 40 seconds at a

constant and freely flow rate with the nozzle placed into the external auditory meatus. During each irrigation, water was collected and its volume measured (range 500 - 600 ml). The temperature of the irrigation water, which was contained in a plastic bucket, was regulated by a thermostatically controlled, heated circulation pump connected to the outlet nozzle by a thick walled rubber tubing. Water was precirculated to ensure a constant temperature from the bucket to the tympanic membrane. The temperature at the nozzle outlet was checked to be $44^{o} \pm 0.5^{o}C$ before each irrigation.

The sequence of testing was always the right ear first and then the left ear. A five minute interval between the end of the first response and the start of the second irrigation allowed the subject to return to resting state. During each test, subjects were given a serial subtraction to do appropriate to their mental abilities. This they were required to do in a soft audible voice to maintain the state of alertness so necessary for a consistent response (Collins, 1962). At the end of the response lights were turned on and the lenses of the Frenzel glasses opened. Calibrations were repeated for the second irrigation.

Each subject was re-tested on two further occasions. Test 2 was performed two to three days after the first test and Test 3 was done three months later.

2.2.5 Calculations

Maximum velocity of the slow phase and maximum frequency of the induced horizontal nystagmus were calculated by dividing each trace into 10 second intervals. For each 10 second interval slow phase velocity was calculated as the mean of

10 consecutive beats and frequency was determined by counting the total number of beats.

2.3 Results

22 of the 25 subjects were tested on two occasions and 18 on three. After the first test, three subjects were excluded from further participation in the study due to lack of co-operation. Four subjectswere unavailable for the third test.

The data on the 18 subjects for the two parameters, maximum slow phase velocity (Table 1) and maximum frequency (Table 2) were analysed, in the first instance, to determine whether sex, age or volume of water used significantly contributed to variation in the data. Analysis of variance was used for this and it was found that none of the three factors proved to be significantly useful explanatory variables.

Further analysis of the data was performed using the Student t-test where the comparisons being made were the difference between the tests, i.e. $T_1 - T_2$, $T_2 - T_3$ and $T_1 - T_3$, for both the right and left ears for each of the two parameters. A similar analysis of the difference between ears, i.e. right - left, for Tests 1, 2 and 3 for both the maximum slow phase velocity and maximum frequency was also performed.

The results of these analyses are given in Tables 3a - c where the mean difference, the standard error of the mean and the Student t statistic are quoted in each case. Graphs of the means on which these tests are based are given in Figures 2 and 3. These results showed that there was a significant reduction in the maximum slow phase velocity in both ears for the group as a whole between Tests 1 and 2 which was sustained at Test 3 three months later (Fig. 2). On the other hand,

Table 1

Individual values obtained for the maximum slow phase velocity (degrees/second) of the three tests

Subject		Age (yrs)	RIGHT EAR Tests 1	2	3	LEFT EAR Tests 1	2	3
1	VA	30	8.2	6.5	4.3	7.7	8.2	4.9
2	TA	37	26.1	18.4	17.8	20.4	22.9	28.4
3	JB	27	23.0	16.4	19.0	15.5	10.4	15.3
4	AB	54	34.5	28.6	18.1	21.5	17.2	13.0
5	FC	60	68.4	33.0	29.9	60.1	36.0	42.7
6	ME	50	32.7	21.8	16.4	17.7	26.7	16.6
7	JH	27	27.4	25.6	10.6	53.3	27.4	29.1
8	JH	34	32.7	19.8	21.1	27.0	30.8	29.5
9	PH	38	59.3	29.9	23.1	55.6	41.7	49.2
10	SH	35	42.3	18.7	17.2	17.4	13.8	14.0
11	RK	41	81.9	52.0	13.6	20.6	17.3	22.2
12	SM	22	14.3	7.6	14.2	20.6	15.0	19.3
13	GN	44	29.0	35.7	31.2	28.3	23.5	30.4
14	PR	35	29.2	14.7	38.4	26.1	17.6	18.9
15	TR	43	29.9	19.2	9.6	17.6	10.3	12.3
16	PS	41	28.5	15.6	10.6	31.1	20.6	17.6
17	DV	20	66.5	31.4	41.2	23.9	20.8	22.5
18	IW	46	9.7	12.8	7.5	17.3	11.2	7.7

Table 2

Individual values obtained for the maximum frequency (beats/second) of the three tests

		RIGHT EAR			LEFT EAR		
		Tests			Tests		
Subject		1	2	3	1	2	3
1	VA	1.3	1.6	1.4	1.1	1.5	1.7
2	TA	2.6	2.3	2.9	3.1	3.0	2.9
3	JB	1.9	1.7	1.7	1.9	1.8	2.4
4	AB	2.6	2.6	2.7	2.4	2.2	2.4
5	FC	3.4	3.1	3.5	2.8	3.2	3.4
6	ME	2.9	2.9	3.8	3.7	4.0	3.8
7	JH	2.4	2.3	2.1	2.9	2.5	2.8
8	JH	2.6	3.0	2.7	3.2	3.5	3.4
9	PH	1.8	1.8	2.1	2.4	2.5	2.1
10	SH	3.1	2.0	2.3	2.8	2.5	2.7
11	RK	2.7	2.5	2.0	2.5	2.0	2.3
12	SM	2.4	2.2	3.0	2.7	2.2	2.9
13	GN	2.8	2.6	2.6	3.0	2.6	2.7
14	PR	2.0	1.8	2.1	2.5	1.7	2.3
15	TR	3.3	3.1	3.3	3.1	2.8	3.9
16	PS	3.2	3.3	2.8	3.2	1.9	3.4
17	DV	4.2	3.7	3.9	3.4	3.6	3.7
18	IW	1.7	2.2	2.0	2.0	2.4	2.2

Table 3a

Comparison of the difference between tests for the right ear using Student's t-test

Parameter	Test	Mean	S.E	t
Maximum Frequency	$T_1 - T_2$	0.13	0.07	1.75
	$T_2 - T_3$	-0.13	0.10	-1.35
	$T_1 - T_3$	0.00	0.10	0.00
Maximum Slow Phase Velocity	$T_1 - T_2$	13.31	2.88	4.62***
	$T_2 - T_3$	3.32	2.64	1.26
	$T_1 - T_3$	16.44	4.22	3.90***

*** $P < 0.001$

Table 3b

Comparison of the difference between tests for the left ear using Student's t-test

Parameter	Test	Mean	S.E	t
Maximum Frequency	$T_1 - T_2$	0.09	0.08	1.14
	$T_2 - T_3$	-0.21	0.09	2.26*
	$T_1 - T_3$	-0.12	0.08	1.51
Maximum Slow Phase Velocity	$T_1 - T_2$	7.54	2.08	3.63**
	$T_2 - T_3$	-1.48	1.15	1.28
	$T_1 - T_3$	5.47	1.79	3.06**

* $P < 0.05$

** $P < 0.01$

Table 3c

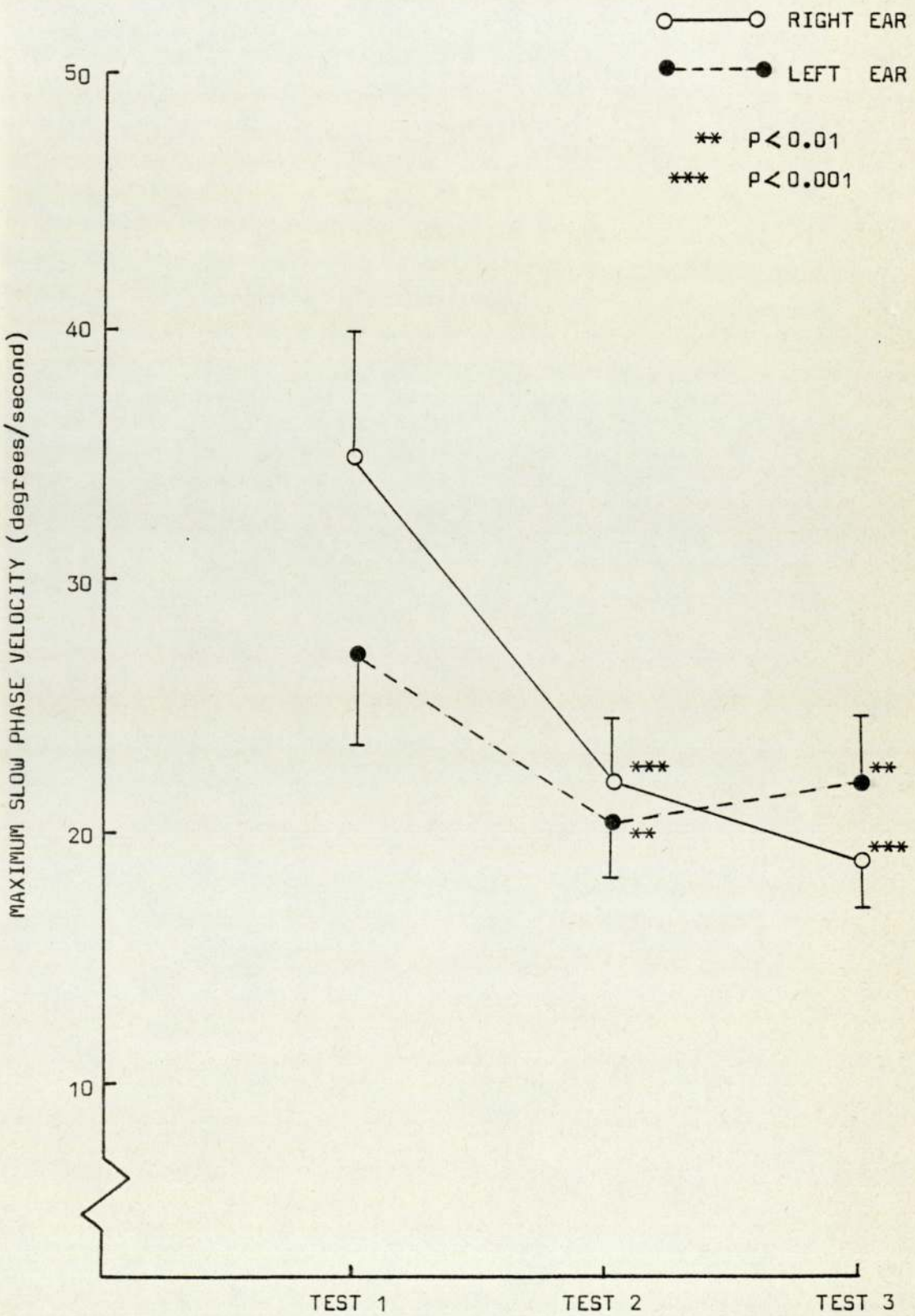
Comparison of the difference between the right and left ears for the three tests using the Student's t-test

Parameter	Test	Mean	S.E	t
Maximum Frequency	T_1	-0.03	0.10	-0.30
	T_2	-0.08	0.10	-0.84
	T_3	-0.22	0.08	-2.92**
Maximum Slow Phase Velocity	T_1	9.06	4.96	1.83*
	T_2	3.83	2.93	1.31
	T_3	-3.09	2.52	-1.23

* $P < 0.05$

** $P < 0.01$

Fig. 2 Group mean responses for the maximum slow phase velocity.



for maximum frequency (Fig. 3) the response was reduced significantly in the right ear at Test 2 but both ears produced equal or greater responses at Test 3 compared to Test 1.

However, when individual subject response values were assessed, it was found that two subjects (subjects 7 and 11) showed large unilateral (right ear) progressive reductions in both the maximum slow phase velocity and maximum frequency at Test 2 and were sustained at Test 3 (Table 4). The maximum percentage difference for maximum frequency was 25.9% and for the maximum slow phase velocity was 85.4%. These differences were shown by subject number 11.

Fig. 3 Group mean responses for the maximum frequency.

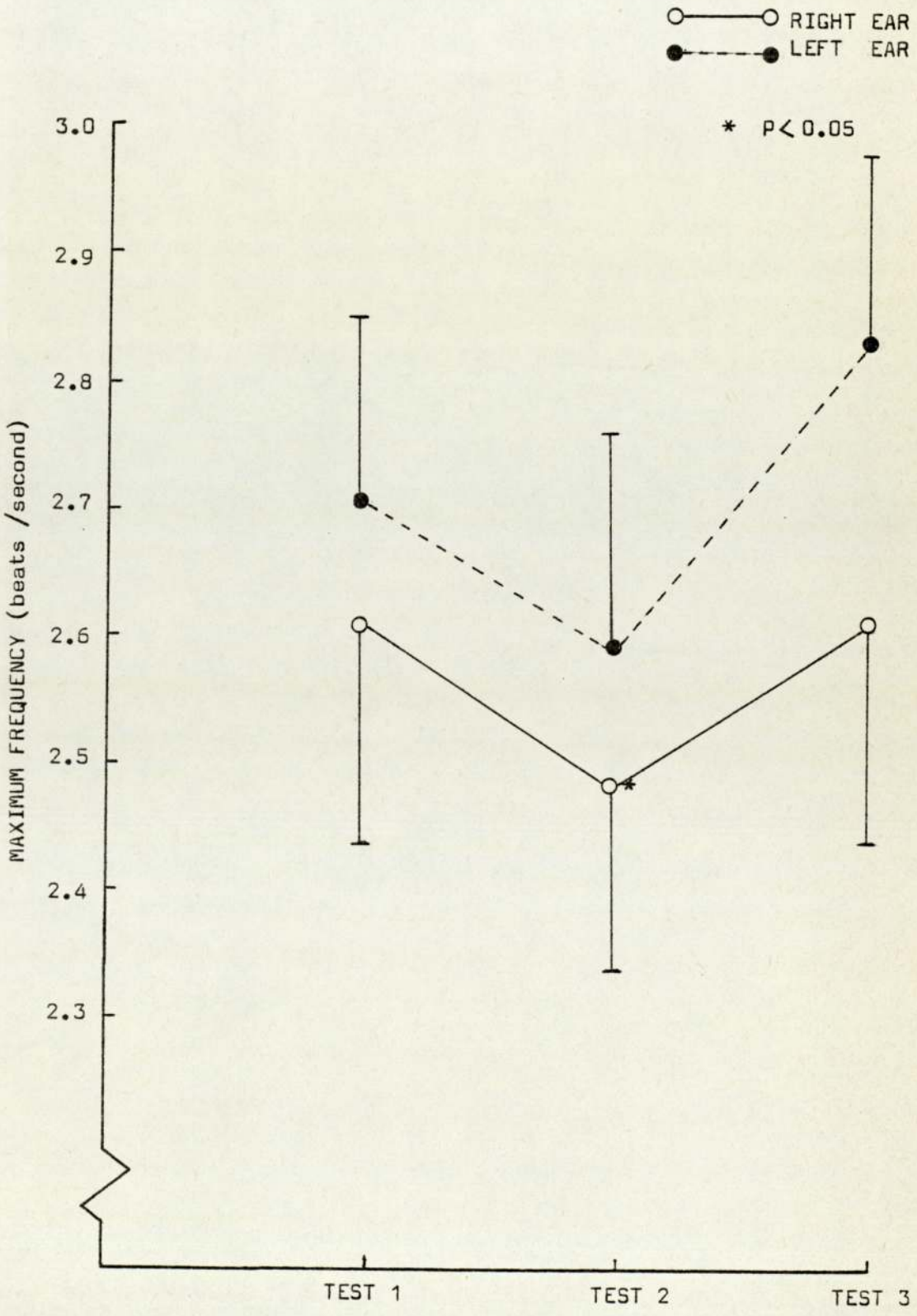


Table 4

Right ear responses from two individuals who showed sustained reductions at Test 3

Subject 7	Maximum Frequency	2.4	2.3 (4.2%)	2.1 (12.5%)
	Maximum Slow Phase Velocity	17.4	15.6 (10.3%)	10.6 (39.1%)
Subject 11	Maximum Frequency	2.7	2.5 (7.4%)	2.0 (25.9%)
	Maximum Slow Phase Velocity	81.9	52.0 (36.5%)	13.6 (83.4%)

2.4 Discussion and Conclusions

The natural history of aminoglycoside vestibular ototoxicity in humans is unknown. In animals aminoglycosides have been shown to produce bilateral histological and functional changes in both the cochlea and semi-circular canals (Wersäll, Lundquist and Björkroth, 1969). It cannot, however, be assumed that aminoglycosides would produce symmetrical functional decrease in both labyrinths in humans. A test for detecting aminoglycoside toxicity must therefore be capable of detecting symmetrical and asymmetrical changes.

The results of this study showed that a significant reduction in the maximum slow phase velocity occurred in both ears for the group as a whole between Tests 1 and 2, which was sustained at Test 3 three months later. For the maximum frequency, on the other hand, only the right ear's responses were reduced significantly at Test 2 and both ears produced equal or greater responses at Test 3 compared to Test 1. For the mean data it can therefore be stated that a reduction in maximum frequency sustained for three months after initial testing would be significant in one or both ears but a reduction in maximum slow phase velocity would not.

The incidence of symptomatic vestibular toxicity has been quoted at 2% for a large retrospective series (Jackson and Arcieri, 1971) and 10% for a prospective series with a preponderance of elderly patients with abnormal renal function (Nordström et al., 1973). The incidence of asymptomatic decrease in vestibular function in Fee et al.'s study (1978) was 11%. Any study which attempts either to determine incidence of toxicity or to compare ototoxicity of two or

more aminoglycosides should therefore have large numbers in each group if mean data are to be used. The alternative is to assess each patient individually to monitor individual's therapy to avoid toxicity. If such an approach is used with the results in this study, it is found that two subjects (numbers 7 anf 11) showed large reductions in both the maximum slow phase velocity and maximum frequency at Test 2 which were sustained at Test 3. Both these reductions were in the right ear only. For individual results it can therefore be stated that sustained, bilateral reductions in maximum slow phase velocity and maximum frequency did not occur but that sustained unilateral reductions in both parameters occurred in two subjects. The occurrence of a reduced response in 11% of the subjects in this study makes assessment of unilateral reductions in vestibular function impossible in patients receiving aminoglycosides tested as outlined.

This phenomena of short and long term reductions in response to caloric irrigation has been previously studied. Two papers are of particular relevance to the results of the present study. Following repeated daily unilateral irrigations with water at 30°C, Lidvall (1961) showed that the caloric response, as assessed by total number of nystagmus beats, was significantly reduced. This decrease was sustained on retesting two weeks later. This response decline could be prevented by making the subjects perform mental tasks (Lidvall, 1962) which caused sufficient alertness to increase galvanic skin resistance (GSR). However, tasks such as simple counting, which did not increase GSR, had no effect on caloric response. Collins (1965) performed repeated unilateral irrigations at

short and long intervals, with water at 30°C on a group of volunteers. Subjects were made to perform sophisticated alerting tasks but nonethless, when tested with eyes open in light, a significant reduction in maximum slow phase velocity was observed with repeated testing which was sustained after an interval of four weeks. Performing the tests with eyes open in darkness produced consistent responses with no significant reduction in maximum slow phase velocity.

The studies by Lidvall and Collins show that short and long term habituation can be abolished by either ensuring that the subject is fully alert or by performing tests on subjects in total darkness. These criteria make a study of sick patients on a ward impossible, since neither subject alertness nor darkness can be relied upon under these conditions. The present study shows the importance of examining the reliability of a technique in a control group of subjects before applying it to a study of patients. It is suggested that workers studying aminoglycoside toxicity with repeated caloric irrigation must also demonstrate that their test-retest variability is acceptable in a comparable group of patients, for instance patients with infections treated with other types of antibiotics tested under the same conditions as the aminoglycoside group.

SECTION 3

STUDIES ON THE RELIABILITY OF AUDIOMETRY

3.1 Introduction

Audiometry was designed to reveal accurate information on the acuity of hearing. The value of this information is dependent on the tester conducting the tests, the subject and the audiometer. The tester must consider a number of variables which, if ignored, may affect the stability of threshold measurements. The co-operation and attention of the patient are essential as in all subjective tests, and his reactions must be observed at all times during the test.

It is generally accepted that the results of clinical pure tone audiometry, if conducted under standard conditions by a trained tester using standardized audiometers and a careful technique under clinical conditions, have a considerable degree of reliability (by reliability is meant the consistency of two or more consecutive audiograms of the same subject).

Some of the variables which may affect threshold values are discussed below:

a. Technique of measurement

Some testers establish threshold values by going from an inaudible to an audible stimulus (ascending method), while others approach the threshold by going from an audible to an inaudible stimulus (descending method). Carhart and Jerger (1959) compared the ascending and descending audiometric techniques to evaluate the extent to which these sequences of stimulus presentation would affect the threshold level. The subjects used were 36 university students whose ages ranged from 18 to 24 years. They found that the mean thresholds obtained by the

descending method were better than the ascending method. The mean differences however, were considerably smaller than 5dB which is the normal intensity interval of clinical audiometers.

b. Instructions to the subject

Another source of variability lies in the instructions given to the subject. If he is told to respond to stimulus even though it is so faint that he may not be completely certain that he heard it, the subject may give 5dB lower thresholds than if instructed to respond only to tones that he is certain he hears. It is clearly important, therefore, that the subject is always given the same instructions.

c. Positioning of earphones

Care has to be taken to place the diaphragm of the audiometer earphone squarely over the opening of the external auditory canal. Failure to do so may result in poor test-retest reliability.

d. Limitations of the audiometer

Characteristics inherent in the design and calibration of audiometers have an influence on the variability of the threshold. Assuming that two audiometers meet current calibration standards (ISO 1964 - International Organization for Standardization; ANSI 1969 - American National Standards Institute), it is possible to test a single subject with each audiometer in exactly the same manner and to record different thresholds and still allow the audiometer to meet current calibration standards (ISO 1964; ANSI 1969). The sound pressure level at each earphone reading of the hearing level (HL - is defined as the number of dB a sound exceeds the normal sound pressure level at that frequency) may differ from the indicated value

by ± 3dB for frequencies 250 to 3000 Hertz (Hz) inclusive, by ± 4dB for 4000Hz, and by ± 5dB for frequencies below 250 and above 4000Hz.

This makes it possible for one audiometer with acceptable specification to deliver 55dB at 8000Hz tone with a dial reading of 50dB HL, and another with acceptable specification to deliver only 55dB of the same tone with a dial reading 60dB HL. If the 'true threshold' was 55dB, then the tester would obtain a dial reading of 50dB on the first audiometer and 60dB on the second. One would then have to record the thresholds as being 10dB different when in fact they were the same.

e. Subject variability

Variability in threshold measurements often relates to the subject being tested. Three factors that contribute to variability of threshold may be:

i. Pathology of the ear.

ii. Motivation of the subject.

iii. Ability of the subject to co-operate.

Certain kinds of ear pathology are associated with physical discomfort. If the patient has Menieres disease and is dizzy or nauseated while being tested, then the recorded thresholds may be higher than when the subject is physically well.

The type of hearing pathology which produces episodic tinnitus might also affect results. Thresholds obtained during the tinnitus attack may be higher than during remission, particularly if there is a similarity between the tone of the tinnitus and the test frequency.

Individuals who have been exposed to high levels of industrial noise or to the sound of gunfire may suffer temporary

shifts of threshold. If so, they will exhibit higher thresholds immediately after the cessation of noise than after a sufficient interval of quiet.

Motivation is also a factor in test reliability. Some individuals, particularly the elderly and infirm, have limited ability to co-operate. They tire easily and have difficulty in maintaining attention during the listening task. Others may not have the intellectual capacity to maintain sustained interest in the test procedure. The tester has to be alert to these possibilities and to be prepared to modify the approach as circumstances require.

f. Ambient noise conditions

Failure to control or to make allowance for one or any combinations of these variables can lead to spurious measurements of threshold. Some variables are more important than others, but in general the more rigidly the testing situation is controlled, the more reliable and valid the measurements are likely to be.

In clinical audiometry it is rarely possible to impose the rigorous experimental controls which might be applied in the laboratory. Clinical audiograms, therefore, would not be expected to be as reliable as laboratory measurements.

3.1.1 Objectives of this study

This study was undertaken to establish the extent of variability which may be encountered in obtaining an audiogram under three sets of conditions. Three separate experiments were carried out:

1. To study the variation which may occur between hearing threshold levels (HTLs) for a subject tested twice under

similar conditions but each time using a different audiometer.

2. To study the variation which may occur between HTL for a subject tested twice on the same audiometer, once on the ward and once in the booth.

3. To determine whether a subject is affected by practice and tends to produce a lower HTL when a repeat measurement is carried out (under identical conditions) the next day.

In all cases both air and bone conduction measurements were studied.

The effect of each of the above variables on HTL was assessed because in the clinical ototoxicity study (Section 4, Page 91) sick patients were tested on the ward using the portable audiometer. Subsequently, the follow-up tests were conducted in the booth using a different audiometer in the ENT Department. On the ward there was a lot of background noise which was not continuous and unvarying over long periods of time. When audiometry is performed on sick patients they are not co-operative firstly due to their illness and secondly they have to concentrate harder to listen to the test tone because they have to compete with the background noise on the ward. It was not known whether the tests performed on the ward could be compared with the tests performed in the booth. If they were not comparable, then should all tests be conducted in the same situation. Also, since the tests were done regularly, then due to familiarization effects, an improvement in apparent hearing levels may mask the real deterioration.

Obviously, all these factors can operate together and combine to produce a large residual variation that any genuine shifts in threshold may be completely masked. Thus, the results of serial audiometry may not be identical on each occasion if

no deterioration has occurred.

In order to evaluate the influence of each of the above sources of possible variability on HTL, healthy subjects although not necessarily having normal hearing thresholds were studied. An attempt was made to eliminate the subjective factors (Section 3.1) which may enter into these experiments involving determination of HTL. Intelligent, co-operative (i.e. reliable) hospital personnel mainly doctors and laboratory technicians were used but if any ear pathology existed, then they were not allowed to participate in the tests.

3.2 Methods and Materials

3.2.1 Subjects

24 male and 24 female members of staff of East Birmingham Hospital were used as subjects. The majority of the subjects (N = 48) were between 19 - 30 years of age; the average age was 28.7 years and the range was 19 - 63 years. No screening procedure was employed and the only criterion used for inclusion in the study was no previous experience with audiometry. Attention was paid to the possibility of the subjects developing rhinitis, either coryzal or allergic in origin, during the course of the experiment from either of these conditions.

3.2.2 Testing procedure

Before the test was performed, the subject was positioned properly and instructed in the listening task. It was explained to the subject that the purpose of the test was to measure the faintest sound (threshold) that he could hear over a range of frequencies and that the accuracy of the results depended upon his willingness to co-operate. He was then seated either in the sound-proofed booth or seated in a position (if the test was being conducted on the ward) where the control panel of the audiometer was out of his line of vision. The method of pressing the switch to show whether or not he heard the tone was described to him. The general pattern of instructions were:

> 'As soon as you hear a tone, press the button. Keep it pressed as long as you hear the tone, no matter which ear you hear it in. Only release the button when the tone goes away. No matter how faint the

tone, press the button when you hear it, and release it as soon as the tone goes away.'

These instructions were accompanied by appropriate simultaneous gestures illustrating and reinforcing the verbal explanation.

Before the earphones were placed over the subject's ears, spectacles and earrings were removed. The ear canals were inspected and any cerumen (wax) was removed. The earphones were then placed in such a position so that the centre of each of the two earphones was in line with the centre of each ear canal. Both earphones pressed lightly against the ear to form a good seal and were held in place by tightening the headband. The earphones on both the audiometers were colour coded so that the red coloured earphone was always placed on the right ear and the blue one on the left ear.

All audiograms were obtained by one tester. In all subjects the left ear was tested first. The results of previous audiograms in the series were not available during subsequent tests; only when the entire study was completed were the audiograms of individual subjects compared.

The 'up 5 - down 10' method of threshold exploration (Coles, 1978) was used. Seven individual frequencies which are normally employed in the clinic were used to obtain the threshold values.

For air-conduction tests, the steps used to obtain HTL (Coles, 1978) are as follows:

1. The test is started with a 1000Hz tone at a level estimated to be sufficiently above threshold to allow easy identification of the tone. This frequency was selected because it is an important speech frequency

and the subject is less apt to misunderstand the listening task.

2. The subject's understanding of the listening task is checked by using both long (2 seconds) and short (0.5 second) duration presentations of the initial test tone. The button should be pressed immediately on presentation of the tone and released as soon as the tone is discontinued. If the movements are sluggish, the subject is reinstructed and encouraged to be more responsive. During the course of testing, the interval between the tone presentations is varied (approximately 0.5 - 2 seconds) to avoid anticipating the stimulus.

3. The starting intensity of the tone is reduced in 10dB steps following each positive response until a hearing threshold level is reached at which the subject fails to respond. Then, the tone is raised 5dB. If the subject hears this increment, the tone is reduced 10dB; if he does not respond, the tone is increased in 5dB steps until it is heard. The essence of the '5 up - down 10' method is that the 5dB increment is always used if the preceeding tone is not heard, and a 10dB decrement is always used when the tone is heard. Threshold is defined as the faintest tone that can be heard 50% or more of the time and is established after several threshold crossings.

4. After the threshold for 1000Hz has been established, the procedure is repeated for the other frequencies in the order 2000, 4000, 6000, 8000, 500, 250 and 1000 (repeat) Hz.

Using the same technique, the test is repeated on the second ear. The bone conduction tests were then performed on

both ears using a bone vibrator. This vibrator (Philips radioear, Model B-70A) was placed on the frontal bone in each case and the thresholds were obtained as above starting with the 1000Hz tone and proceeding to 2000, 4000, 500 and lastly 1000Hz.

All the above tests were administered with either the Kamplex (Model TA 155) or the Amplivox (Model 103) clinical diagnostic audiometers using TDH-39 earphones fitted with MX-41/AR supra-aural cushions. The Kamplex audiometer being a portable audiometer had audio-cups which were fitted over the earphones and the cushions to reduce the ambient noise.

3.3 Experiment I

The reliability of threshold determination using two different audiometers

12 male and 12 female subjects were tested with each of the two audiometers (Section 3.2.2) in the sound-proofed booth using the procedures described earlier (Section 3.2.2). To analyze the variability between the two audiometers, a crossover experimental design (Cochran and Cox, 1957) experiment was employed. Each subject was tested twice; once with each of the audiometers. The second test was performed 10 minutes after the first test had been completed. In this crossover method, the subjects were tested with each of the audiometers as follows:

Subject 1 would be tested with the Kamplex audiometer followed by the Amplivox.

Subject 2 would be tested with the Amplivox first followed by the Kamplex and so on.

From an experiment of this type, the difference between the two audiometers could then be determined without biasing the results whether the tests were conducted on each of the audiometers before or after the other audiometer.

3.3.1 Results

The HTLs obtained with the two audiometers for both the air and bone conduction tests are shown in Tables 5 and 6. A number of problems arise in the analysis of these data. In analysing these data one can either compare the left ears with left ears and right ears with right ears or an average of both ears. There may be a difference between the HTLs of the left and right ears and between the sound pressure level at each earphone. Consequently, the results have been analysed on the basis of a comparison of the averaged HTL of each ear separately.

The mean hearing threshold levels for the left and right ears of the 24 subjects measured on the two audiometers are shown in Figures 4 (air conduction) and 5 (bone conduction). Paired t-test showed that there were no significant differences between the mean threshold levels obtained with the two audiometers at all frequencies for both the left and right ears with both the air and bone conduction tests (Tables 7 and 8).

The data were further subjected to graphical comparisons. Although graphs were plotted for the left and right ears at each frequency for both the air conduction and bone conduction tests, graphs for the 250Hz frequency of the air conduction tests are only shown here for clarity. Each graph and histogram represents the measurements for 24 ears. The distributions of the HTL obtained with the two audiometers for each frequency were plotted (Fig. 6). For each frequency a scatter diagram was plotted to show how the hearing threshold levels obtained on the two systems agreed (Fig. 7). Below each graph a histogram was plotted showing the number of times a particular

Table 5

Data obtained from 24 subjects using the two audiomters for air conduction tests. The hearing losses in decibels for the same ear on the two systems at each frequency are shown listed side by side

	SUBJECT	AGE (yrs)	HEARING THRESHOLD LEVEL (HTL) IN DECIBELS (dB) 250Hz A	250Hz K	500Hz A	500Hz K	1000Hz A	1000Hz K	2000Hz A	2000Hz K	4000Hz A	4000Hz K	6000Hz A	6000Hz K	8000Hz A	8000Hz K
1	Left ear	23	5	5	5	0	10	5	5	0	5	5	15	15	20	20
	Right ear		5	15	10	10	5	0	5	0	10	10	10	10	5	10
2	Left ear	24	10	10	5	10	5	5	5	10	10	5	30	40	20	25
	Right ear		10	5	10	10	5	5	15	10	15	10	15	20	25	35
3	Left ear	25	10	10	10	5	10	10	10	5	10	5	25	30	10	15
	Right ear		25	25	15	15	10	15	5	10	5	0	30	25	30	30
4	Left ear	21	10	10	0	0	5	5	0	0	5	0	5	5	-5	5
	Right ear		0	10	0	10	0	0	0	0	15	15	10	10	15	10
5	Left ear	19	10	10	10	10	5	0	10	10	10	15	25	30	15	35
	Right ear		5	10	10	10	5	0	10	10	5	10	30	30	25	30
6	Left ear	24	15	15	15	15	5	10	10	15	15	5	15	15	10	15
	Right ear		25	25	15	15	15	25	5	5	10	10	15	10	15	10
7	Left ear	35	5	10	0	10	0	0	0	5	5	5	25	30	30	35
	Right ear		5	5	0	0	-10	-5	-5	-5	0	5	20	20	5	10
8	Left ear	31	5	10	5	5	10	10	5	5	15	10	10	15	15	15
	Right ear		10	15	5	10	5	10	5	0	10	5	5	15	5	15
9	Left ear	30	25	20	25	25	30	35	20	25	25	30	20	30	35	30
	Right ear		70	65	70	75	70	70	60	60	75	70	85	90	75	90
10	Left ear	38	10	10	5	5	10	5	15	15	35	40	45	50	55	65
	Right ear		10	15	10	15	10	10	10	15	30	40	35	35	35	40
11	Left ear	27	10	5	10	10	5	5	0	0	0	0	10	10	15	20
	Right ear		15	20	20	15	15	10	15	10	15	15	25	25	30	30
12	Left ear	27	15	25	10	10	15	15	15	15	30	30	35	35	35	35
	Right ear		20	20	10	15	15	10	20	20	30	30	25	30	25	30
13	Left ear	25	10	15	5	10	5	10	5	10	10	10	20	20	5	5
	Right ear		10	15	10	15	0	5	5	5	5	10	15	10	0	5
14	Left ear	23	0	10	5	10	5	10	15	15	0	0	10	15	5	10
	Right ear		0	5	5	10	-5	0	20	20	0	0	10	10	5	5
15	Left ear	41	10	15	5	15	10	15	10	15	15	15	35	30	30	30
	Right ear		5	10	15	10	5	10	10	15	30	30	35	35	30	30
16	Left ear	22	5	5	5	10	5	10	-5	0	5	0	15	15	10	10
	Right ear		10	15	5	10	0	5	5	5	0	0	15	20	0	5
17	Left ear	24	15	15	15	20	10	10	15	15	10	10	25	20	15	15
	Right ear		5	5	10	10	5	5	10	10	10	15	20	25	20	30
18	Left ear	24	5	5	0	5	0	0	-5	5	15	10	10	10	5	5
	Right ear		0	5	5	5	0	0	0	5	5	5	10	10	5	5
19	Left ear	22	10	5	10	10	0	5	0	5	5	10	15	20	0	0
	Right ear		5	15	10	15	0	0	0	5	10	5	10	15	10	10
20	Left ear	32	5	10	10	10	5	0	0	0	-5	-5	5	0	0	0
	Right ear		15	10	15	15	5	10	0	0	-5	-5	5	0	0	5
21	Left ear	30	10	10	10	15	5	10	5	10	5	10	25	20	5	15
	Right ear		10	10	5	15	10	10	5	10	10	5	10	20	5	15
22	Left ear	21	5	0	-5	5	-5	5	0	5	-5	-5	5	10	5	10
	Right ear		5	5	0	5	5	5	0	5	0	5	5	20	0	5
23	Left ear	29	15	15	15	15	10	10	0	5	25	25	45	50	55	65
	Right ear		15	20	20	25	25	25	15	20	25	30	50	55	45	45
24	Left ear	28	20	20	25	30	35	35	45	50	55	55	60	60	75	75
	Right ear		30	25	35	25	45	35	40	40	45	40	50	35	55	45

A = Amplivox Audiometer; K = Kamplex Audiometer

Table 6

Data obtained from 24 subjects using the two audiometers for the bone conduction tests

	SUBJECT	AGE (yrs)	HEARING THRESHOLD LEVEL IN dB							
			500Hz		1000Hz		2000Hz		4000Hz	
			A	K	A	K	A	K	A	K
1	Left ear	23	0	5	5	0	0	0	10	15
	Right ear		0	5	-5	-5	-5	-5	10	5
2	Left ear	24	-5	-5	0	0	10	10	15	15
	Right ear		-5	-5	0	0	10	10	15	15
3	Left ear	25	-5	-5	0	0	10	10	15	15
	Right ear		0	-5	-5	-5	10	5	20	20
4	Left ear	21	0	-5	-5	0	-5	0	20	15
	Right ear		0	0	0	0	-5	0	15	15
5	Left ear	19	-5	-5	0	0	10	10	20	25
	Right ear		0	5	0	0	10	10	10	15
6	Left ear	24	-5	0	0	5	10	10	20	20
	Right ear		0	-5	0	5	10	15	25	25
7	Left ear	35	-10	-5	-10	-10	0	0	15	15
	Right ear		-10	-5	-10	-5	5	-5	20	20
8	Left ear	31	-5	-5	-5	-5	5	5	15	15
	Right ear		-10	5	0	-5	5	15	5	10
9	Left ear	30	20	25	20	20	25	25	30	35
	Right ear		40	25	25	20	40	30	45	45
10	Left ear	38	0	-10	-5	-10	10	10	35	35
	Right ear		0	-5	0	-10	10	15	30	30
11	Left ear	27	0	0	-5	0	-10	-5	15	15
	Right ear		5	0	0	0	5	5	25	20
12	Left ear	27	5	10	10	10	10	15	35	40
	Right ear		5	10	10	5	15	20	40	35
13	Left ear	25	0	0	0	0	5	20	20	15
	Right ear		0	5	-5	0	5	10	20	25
14	Left ear	23	-5	-5	-5	-5	25	30	15	20
	Right ear		5	0	0	-5	25	30	25	15
15	Left ear	41	0	15	-5	5	5	5	15	15
	Right ear		-5	0	5	-5	10	10	25	25
16	Left ear	22	0	-5	5	5	10	5	15	10
	Right ear		-5	0	-5	0	5	10	15	15
17	Left ear	24	10	10	5	10	10	10	10	15
	Right ear		5	5	0	5	5	0	15	10
18	Left ear	24	0	0	-5	-5	0	0	15	15
	Right ear		-5	0	-5	-5	-5	0	10	0
19	Left ear	22	-5	0	0	0	5	15	15	15
	Right ear		0	15	-5	0	5	10	15	15
20	Left ear	32	-10	0	5	5	0	5	20	20
	Right ear		-5	5	0	5	0	5	10	15
21	Left ear	30	-5	-5	10	5	10	10	15	15
	Right ear		0	-5	5	0	10	10	15	15
22	Left ear	21	-10	-5	0	0	0	5	15	10
	Right ear		-10	-10	-5	0	0	5	10	20
23	Left ear	29	10	10	5	10	15	15	25	25
	Right ear		20	5	15	0	15	20	30	25
24	Left ear	28	10	5	0	0	0	0	10	10
	Right ear		5	10	5	-5	0	-5	5	10

A = Amplivox audiometer; K = Kamplex audiometer

Fig. 4 Mean hearing threshold levels for the right and left ears of 24 subjects measured on the two audiometers - air conduction tests.

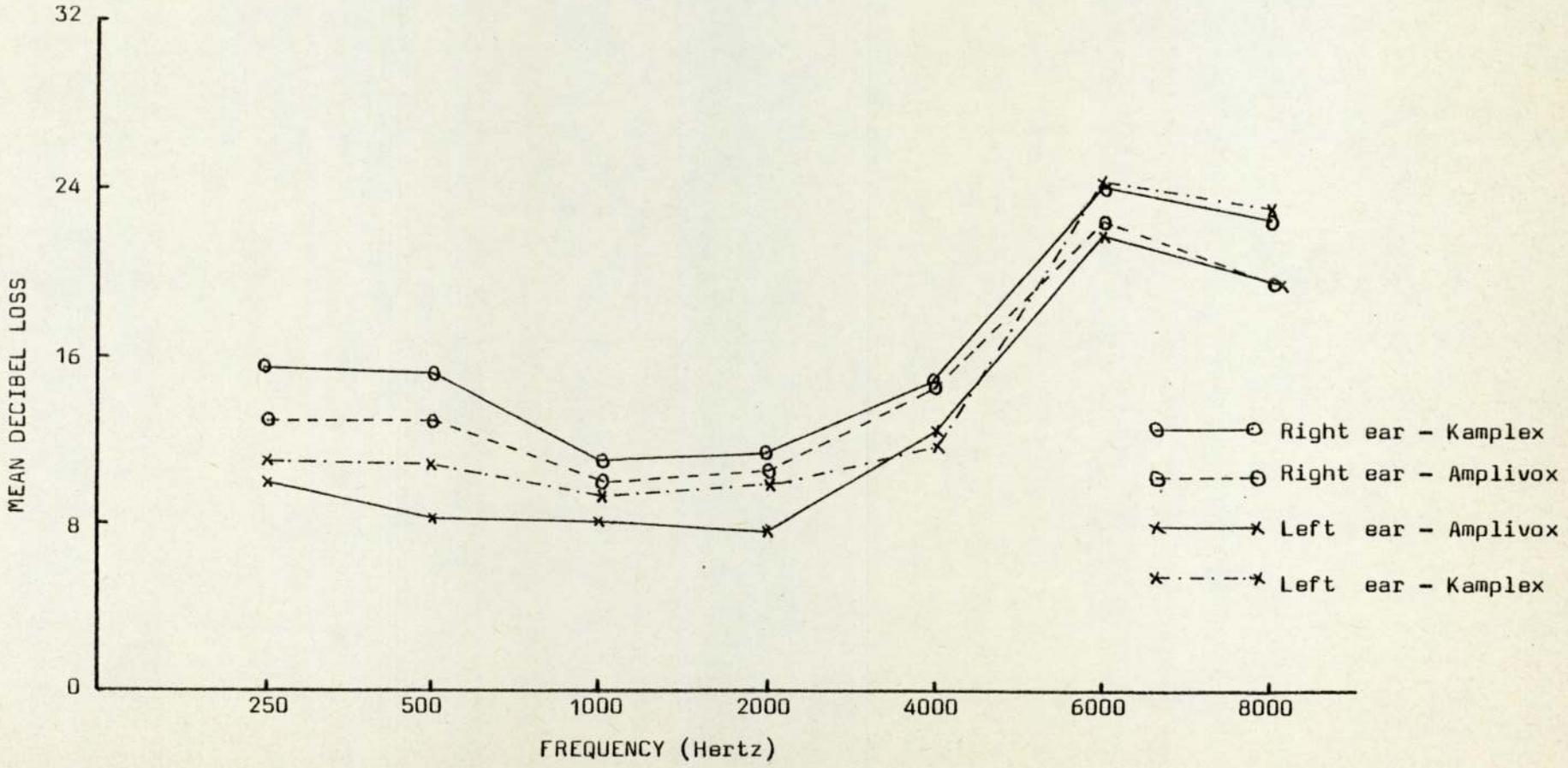


Fig. 5 Mean hearing levels for the right and left ears of 24 subjects measured on the two audiometers - bone conduction tests.

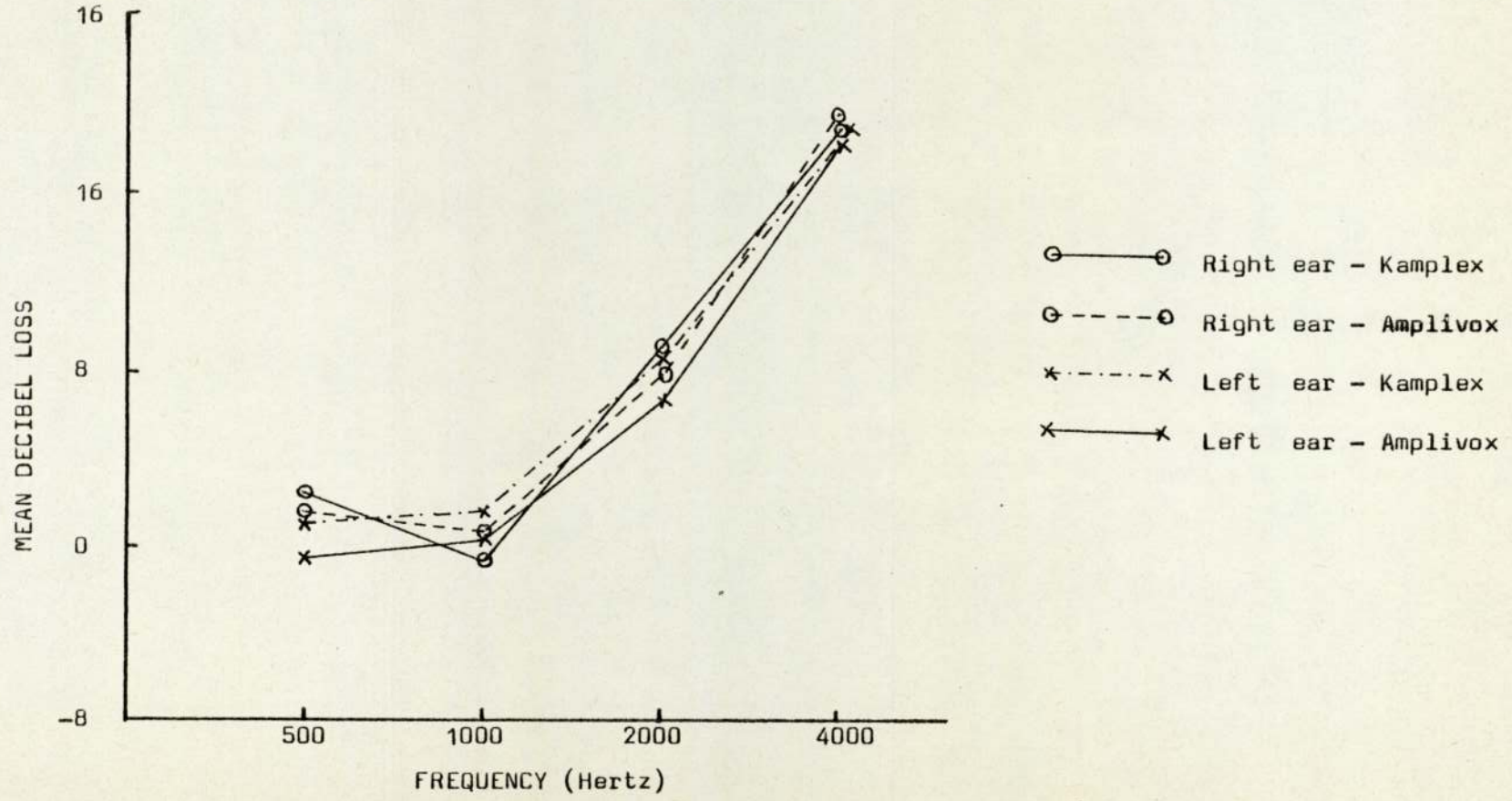


Table 7

Comparison of data obtained for the air conduction tests with the two audiometers

Frequency (Hz)	Ears (N=24)	Mean Value Kamplex	Standard Error Kamplex	Mean Value Amplivox	Standard Error Amplivox	t Value	* r
250	Left ear	11.0	2.2	10.0	2.0	-1.23	0.73
	Right ear	15.4	3.1	12.9	2.6	-2.63	0.95
500	Left ear	10.8	2.2	8.3	1.7	-2.94	0.84
	Right ear	15.0	3.1	12.9	2.6	-2.20	0.95
1000	Left ear	9.4	1.9	8.1	1.7	-1.54	0.92
	Right ear	11.0	2.2	10.0	2.0	-0.89	0.96
2000	Left ear	10.0	2.0	7.9	1.6	-3.12	0.95
	Right ear	11.4	2.3	10.6	2.2	-1.16	0.97
4000	Left ear	11.9	2.4	12.5	2.6	0.77	0.96
	Right ear	14.8	3.0	14.8	3.0	-0.24	0.97
6000	Left ear	24.2	4.9	22.1	4.5	-2.10	0.96
	Right ear	24.0	4.9	22.5	4.6	-1.16	0.94
8000	Left ear	23.1	4.7	19.4	4.0	-3.42	0.97
	Right ear	22.7	4.6	19.4	4.0	-2.89	0.96

* r = correlation coefficient

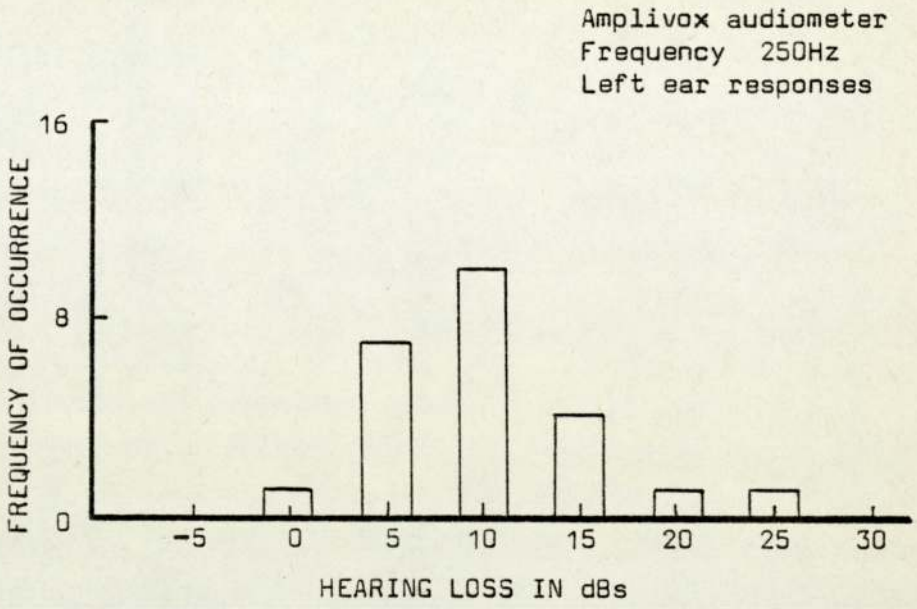
Table 8

Comparison of data obtained for the bone conduction tests with the two audiometers

Frequency (Hz)	Ears (N=24)	Mean Value Kamplex	Standard Error Kamplex	Mean Value Amplivox	Standard Error Amplivox	t Value	*r
500	Left ear	1.0	0.2	-0.6	0.1	-1.37	0.67
	Right ear	2.3	0.5	1.5	0.3	-0.53	0.77
1000	Left ear	1.5	0.3	0.4	0.1	-1.16	0.64
	Right ear	-0.6	0.1	0.6	0.1	1.03	0.86
2000	Left ear	8.3	1.7	6.5	1.3	-2.46	0.83
	Right ear	8.8	1.8	7.7	1.6	-1.14	0.86
4000	Left ear	18.8	3.8	18.1	3.7	-0.62	0.88
	Right ear	18.8	3.8	19.2	3.9	0.42	0.91

*r = correlation coefficient

Fig. 6 The distribution of the hearing threshold levels obtained with the two audiometers - air conduction tests.



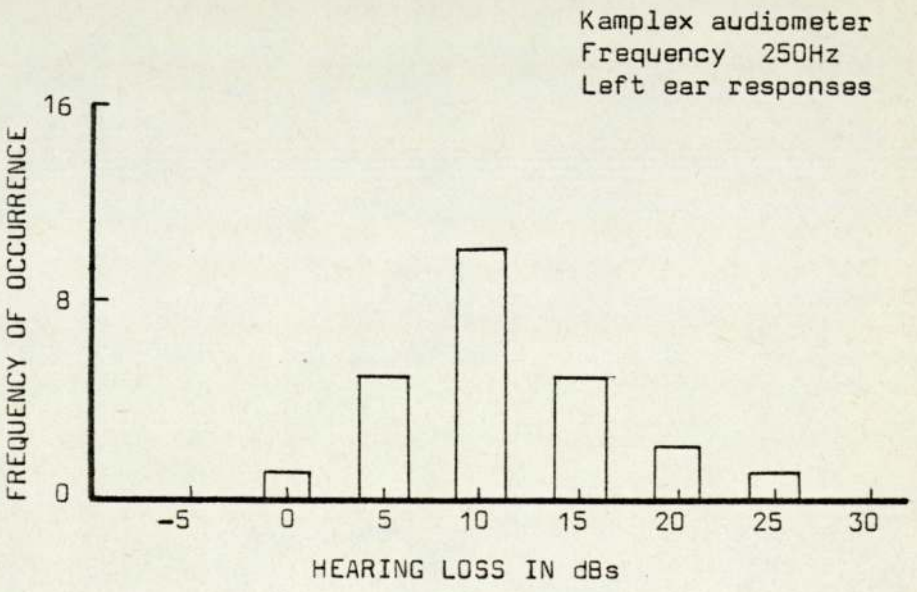
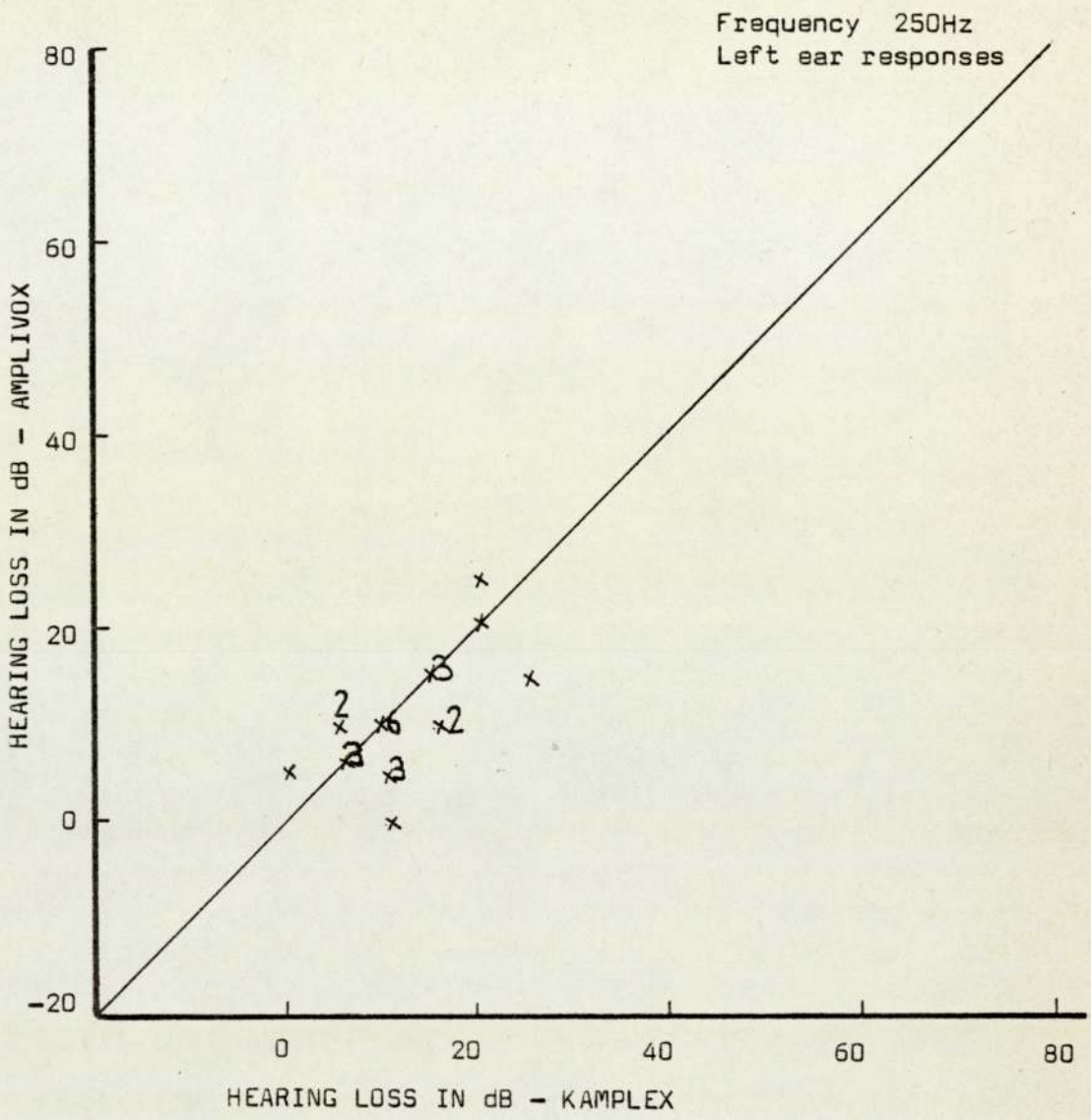
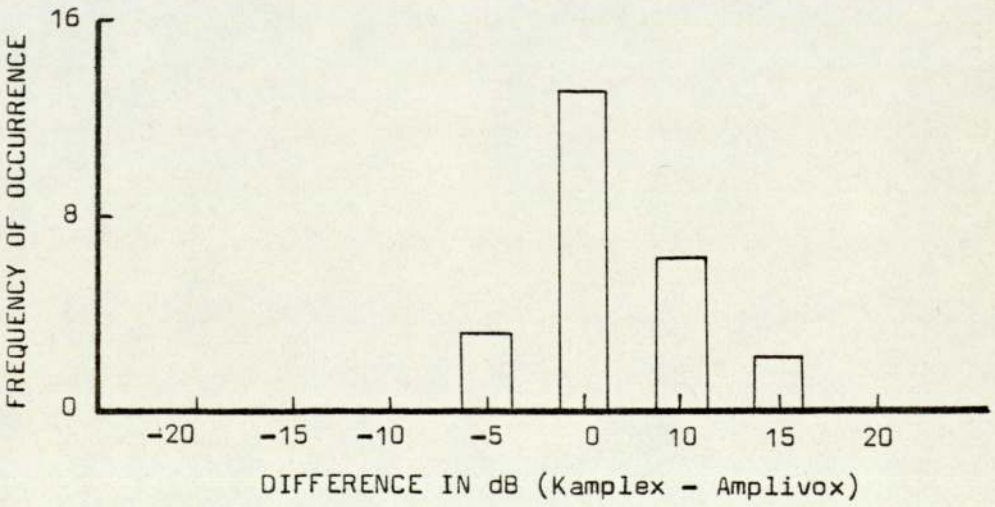


Fig. 7 Hearing threshold levels obtained with the two audiometers - air conduction tests.





difference occurred between the two sets of results at this frequency. With reference to the scatter diagrams, points falling on the diagonal line on each graph represent perfect agreement between the two systems and it can be seen how the results scatter about this line. At all frequencies, there was a tendency for the points to fall below the diagonal line. This indicates that, for the majority of the ears tested, the Amplivox audiometer gave a lower value of the HTL although this was found statistically to be non-significant ($P > 0.05$). In order to show how the agreement between the measurements varied with the test frequency, correlation coefficients (r) were calculated for each frequency, assuming a linear relationship between the two variables. The values for the correlation coefficients are shown in Tables 9 and 10. Examination of the correlation coefficients, using the t-test showed that there was a highly significant correlation between the results of both the air and bone conduction tests.

The most frequently occurring difference was 0dB further proving that the two audiometers were giving similar results (Figs. 8 and 9). With the exception of one frequency (4000Hz; right ear), the mean values of the differences showed that the Kamplex audiometer gave greater threshold values. These values were all less than 5dB and were found to be statistically non-significant for both the air conduction (Table 9) and bone conduction tests (Table 10).

Fig. 8 Differences in hearing threshold levels obtained with the two audiometers - air conduction tests.

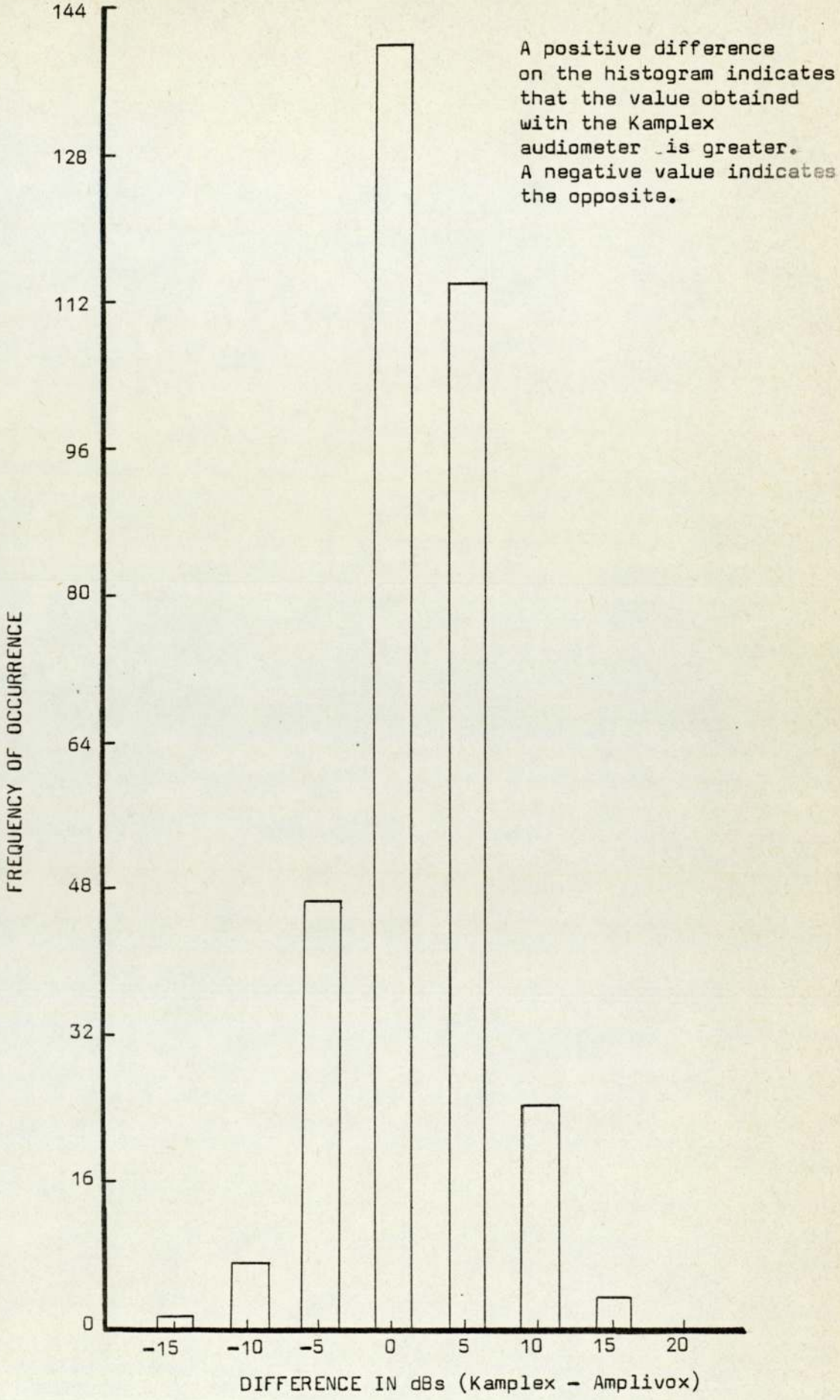


Fig. 9 Differences in hearing threshold levels obtained with the two audiometers - bone conduction tests.

A positive difference on the histogram indicates that the value of HTL obtained with the Kamplex audiometer is greater. A negative value indicates the opposite.

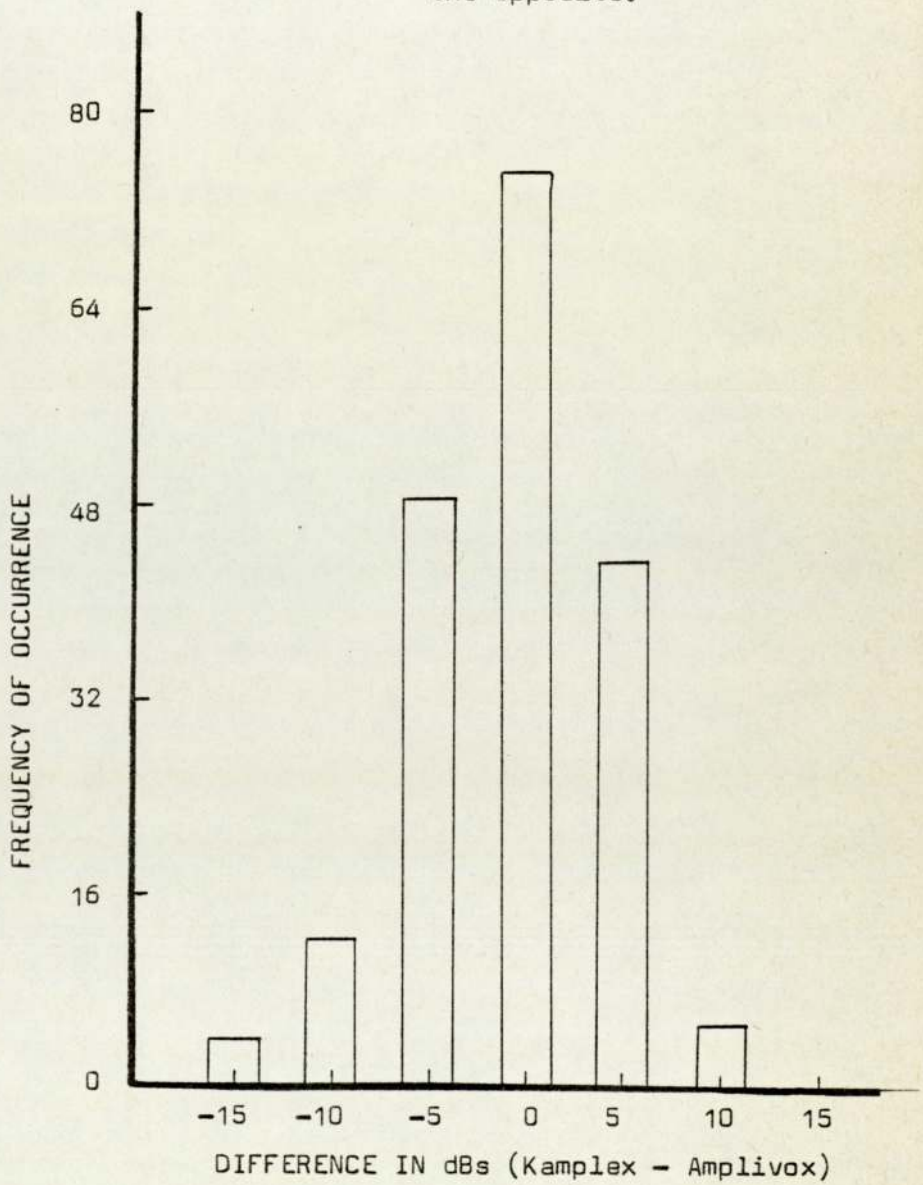


Table 9

Mean values obtained for each frequency with the two audiometers for the air conduction tests

Frequency (Hz)	Ear (N=24)	Mean K (dB)	Mean A (dB)	K - A (dB)
250	Left ear	11.0	10.0	1.0
	Right ear	15.4	12.9	2.5
500	Left ear	10.8	8.3	2.5
	Right ear	15.0	12.9	2.1
1000	Left ear	9.4	8.1	1.3
	Right ear	11.0	10.0	1.0
2000	Left ear	10.0	7.9	2.1
	Right ear	11.4	10.6	0.6
4000	Left ear	11.9	12.5	-0.6
	Right ear	14.8	14.8	0.0
6000	Left ear	24.2	22.1	2.1
	Right ear	24.0	22.5	1.5
8000	Left ear	23.1	19.4	3.7
	Right ear	22.7	19.4	3.3

K = Kamplex audiometer

A = Amplivox audiometer

Table 10

Mean values obtained for each frequency with the audiometers for the air conduction tests

Frequency (Hz)	Ear (N=24)	Mean K (dB)	Mean A (dB)	K - A (dB)
500	Left ear	1.0	-0.6	1.6
	Right ear	2.3	1.5	0.8
1000	Left ear	1.5	0.4	1.1
	Right ear	-0.6	0.6	1.2
2000	Left ear	8.3	6.5	1.8
	Right ear	8.8	7.7	0.7
4000	Left ear	18.8	18.1	0.7
	Right ear	18.8	19.2	-0.4

K = Kamplex audiometer

A = Amplivox audiometer

3.4 Experiment II

The consistency of the threshold determination on the ward compared with the sound-proofed booth

12 male and 12 female subjects were tested with the Kamplex audiometer either on the ward or in the sound-proofed booth. In this experiment a second group of 24 subjects were tested to analyze the variation that may be encountered on conducting the tests on the ward rather than the sound-proofed booth. Crossover method of testing (Cochran and Cox, 1957) was again applied to eliminate any bias which might be introduced by first performing the test always on the ward or always in the booth. Hence, if subject 1 was tested first on the ward then the second test was performed in the sound-proofed booth. Subject 2 was then tested first in the sound-proofed booth followed by being tested on the ward and so on. The time interval between the tests was approximately 15 minutes.

3.4.1 Results

The HTL obtained by conducting the tests on the ward or in the sound-proofed booth for both the air conduction tests and the bone conduction tests are shown in Tables 11 and 12. The HTL for the left and right ears of the 24 subjects measured in both the cases are shown in Figures 10 and 11. Paired t-test showed that there were no significant differences between the mean threshold values, obtained either on the ward or in the sound-proof booth, for frequencies above 1000Hz with the air conduction tests (Table 13). However, for frequencies below 1000Hz there was a significant difference between the ward and the sound-proof booth values for both the ears. On the other hand, the bone conduction tests did not show any significant difference at any frequency for either of the ears (Table 14).

The data were further analyzed by graphical comparisons. In each case the graphs and the histograms represent measurements for 24 ears. For each frequency a scatter diagram was plotted to show how the threshold values obtained in each case agreed (Figs. 12 and 13). Below each graph, a histogram showing the number of times a particular difference occurred between the two sets of results at this frequency was plotted. Points falling on the diagonal line on each scatter diagram represented perfect agreement between the two situations. Although graphs were plotted for all the frequencies, for clarity only the results for the air conduction tests at the frequencies 250 and 500Hz are shown here. For the air conduction tests, it could be seen that at low frequencies there was a tendency for the points to fall below the line (Figs. 12 and 13). This indicated that, at low frequencies the threshold values

Table 11

Data obtained from 24 subjects tested on the ward and in booth using the Kamplex audiometer – air conduction tests

	SUBJECT	AGE (yrs)	HEARING THRESHOLD LEVEL IN DECIBELS(dB) 250Hz W	250Hz B	500Hz W	500Hz B	1000Hz W	1000Hz B	2000Hz W	2000Hz B	4000Hz W	4000Hz B	6000Hz W	6000Hz B	8000Hz W	8000Hz B
1	Left ear	19	15	5	10	10	10	0	10	5	0	-5	15	15	0	-5
	Right ear		10	10	10	10	5	5	5	0	5	10	10	5	15	10
2	Left ear	27	15	10	10	10	10	15	10	5	15	10	15	20	0	0
	Right ear		5	5	5	5	-5	0	5	5	20	15	20	20	25	25
3	Left ear	30	15	5	5	5	5	5	5	0	10	5	10	10	15	5
	Right ear		20	5	10	5	5	5	0	0	10	15	15	10	0	5
4	Left ear	30	15	15	20	20	25	25	20	20	10	15	30	30	30	35
	Right ear		10	10	15	10	10	10	10	15	15	20	15	15	10	10
5	Left ear	29	10	5	10	5	15	15	20	25	30	30	35	35	35	35
	Right ear		15	15	20	10	10	10	15	10	30	30	35	35	25	30
6	Left ear	26	0	5	10	5	5	5	20	15	25	25	10	10	5	5
	Right ear		0	5	10	5	10	0	15	20	20	15	25	15	5	5
7	Left ear	30	15	10	10	10	10	5	5	5	15	15	20	20	20	15
	Right ear		25	10	15	5	10	10	10	10	10	5	20	10	10	10
8	Left ear	30	15	20	10	10	5	5	5	5	15	10	15	15	15	15
	Right ear		15	15	10	15	10	10	10	10	10	15	25	25	20	20
9	Left ear	19	10	10	5	10	10	5	5	5	10	10	15	15	5	10
	Right ear		20	10	15	10	15	15	5	10	5	5	15	10	30	25
10	Left ear	29	20	15	15	10	5	0	5	0	10	10	25	20	20	15
	Right ear		25	20	20	15	5	5	0	0	5	5	25	30	20	20
11	Left ear	36	20	10	15	10	10	5	10	5	10	5	25	25	20	20
	Right ear		20	10	10	10	5	5	10	10	0	0	35	25	25	25
12	Left ear	27	5	5	15	10	5	0	5	5	10	5	10	10	5	10
	Right ear		20	10	15	10	5	10	5	5	10	10	20	15	5	5
13	Left ear	20	10	10	5	10	5	5	0	-5	0	0	10	5	0	5
	Right ear		25	20	20	15	10	10	5	5	10	10	25	20	20	15
14	Left ear	22	10	15	15	15	10	10	10	15	15	25	15	20	20	20
	Right ear		20	10	10	15	5	10	0	5	10	15	5	10	0	5
15	Left ear	31	20	15	20	15	15	15	20	20	30	30	35	35	35	40
	Right ear		25	30	15	15	15	15	15	10	30	15	40	30	35	35
16	Left ear	29	20	15	10	10	5	5	0	0	5	10	15	15	10	25
	Right ear		20	15	15	10	5	10	10	15	5	15	15	10	20	25
17	Left ear	63	10	10	15	10	0	0	5	10	5	0	25	30	10	5
	Right ear		20	15	10	10	5	10	5	0	5	10	20	15	15	25
18	Left ear	28	25	25	15	20	15	15	5	10	30	25	35	30	20	25
	Right ear		25	20	30	25	20	15	20	20	30	30	25	30	25	25
19	Left ear	29	10	10	10	10	5	10	0	0	0	10	15	15	5	5
	Right ear		15	5	15	10	0	0	5	5	5	10	15	15	10	15
20	Left ear	22	5	10	15	15	10	10	10	15	20	20	20	25	5	5
	Right ear		10	10	5	10	10	15	10	15	5	10	20	15	5	5
21	Left ear	24	20	15	20	10	10	10	10	10	20	20	30	30	15	20
	Right ear		25	15	20	15	10	5	5	5	15	15	25	20	15	15
22	Left ear	20	10	5	10	10	5	5	0	0	0	0	10	15	0	5
	Right ear		20	15	10	5	5	0	5	5	10	0	10	15	10	5
23	Left ear	24	15	10	10	5	5	5	10	10	20	20	40	35	30	25
	Right ear		15	10	10	10	5	5	10	5	25	25	15	15	10	10
24	Left ear	21	15	10	10	5	10	10	15	5	10	0	15	10	15	20
	Right ear		15	15	10	10	10	10	10	5	15	10	20	20	15	15

W = Ward; B = Booth

Table 12

Data obtained from 24 subjects tested on the ward and in the booth using the Kamplex audiometer- bone conduction tests

SUBJECT	AGE (yrs)	HEARING THRESHOLD LEVEL IN DECIBELS (dB) 500Hz W	500Hz B	1000Hz W	1000Hz B	2000Hz W	2000Hz B	4000Hz W	4000Hz B
1 Left ear	19	-5	5	0	-5	-5	-5	0	5
Right ear		0	-5	-5	-5	-10	0	10	10
2 Left ear	27	0	0	-5	-5	10	15	20	20
Right ear		0	-5	-10	-10	15	15	20	20
3 Left ear	30	0	0	-5	-5	10	0	15	15
Right ear		0	0	-5	-10	5	5	15	10
4 Left ear	30	5	5	5	0	10	10	20	15
Right ear		0	0	5	5	10	5	20	20
5 Left ear	29	5	-5	-5	0	10	10	25	25
Right ear		-5	0	0	0	5	5	30	30
6 Left ear	26	0	5	10	0	20	25	30	30
Right ear		0	10	10	5	20	25	25	20
7 Left ear	30	-5	0	-5	0	5	5	25	20
Right ear		0	-5	-5	-5	10	5	15	15
8 Left ear	30	0	-5	-5	-5	-5	0	20	15
Right ear		0	-5	0	0	0	5	25	25
9 Left ear	19	5	5	0	0	0	0	15	10
Right ear		10	5	10	10	0	5	0	0
10 Left ear	29	-5	-5	-5	-5	0	0	15	15
Right ear		-5	0	-5	-10	0	-5	25	15
11 Left ear	36	-5	-5	-5	-5	0	5	15	10
Right ear		0	5	0	0	10	10	10	10
12 Left ear	27	5	0	0	-5	5	5	15	10
Right ear		5	10	5	5	5	5	10	5
13 Left ear	20	-5	0	5	0	5	5	15	15
Right ear		5	10	-5	-5	10	5	20	15
14 Left ear	22	15	-5	5	10	10	5	20	25
Right ear		-5	5	10	10	5	5	15	15
15 Left ear	31	0	0	0	0	5	15	30	35
Right ear		5	0	10	5	25	10	35	30
16 Left ear	29	0	-5	0	5	10	10	25	20
Right ear		0	5	0	-5	10	5	20	20
17 Left ear	63	-5	0	-5	-5	5	0	5	5
Right ear		-5	-5	-5	0	-5	-5	10	15
18 Left ear	28	15	10	10	10	15	20	30	35
Right ear		10	10	5	10	5	5	35	30
19 Left ear	29	0	-5	-10	-10	10	15	20	15
Right ear		-5	-10	-5	-5	10	10	20	25
20 Left ear	22	0	-5	5	15	10	15	25	25
Right ear		-5	0	0	10	10	15	15	15
21 Left ear	24	0	-5	0	-5	10	15	25	25
Right ear		0	0	-5	-5	10	5	25	25
22 Left ear	20	-5	5	-5	-5	-5	0	10	15
Right ear		0	0	-5	-5	-5	5	15	15
23 Left ear	24	0	-5	0	-5	5	10	45	45
Right ear		-5	-5	-5	-5	5	10	40	45
Left ear		5	-5	0	0	10	10	25	15
Right ear		-5	0	10	0	15	15	25	20

W = Ward ; B = Booth

Fig. 10 Mean hearing threshold levels for the right and left ears of 24 subjects measured on the ward and in the booth using the Kamplex audiometer - air conduction tests.

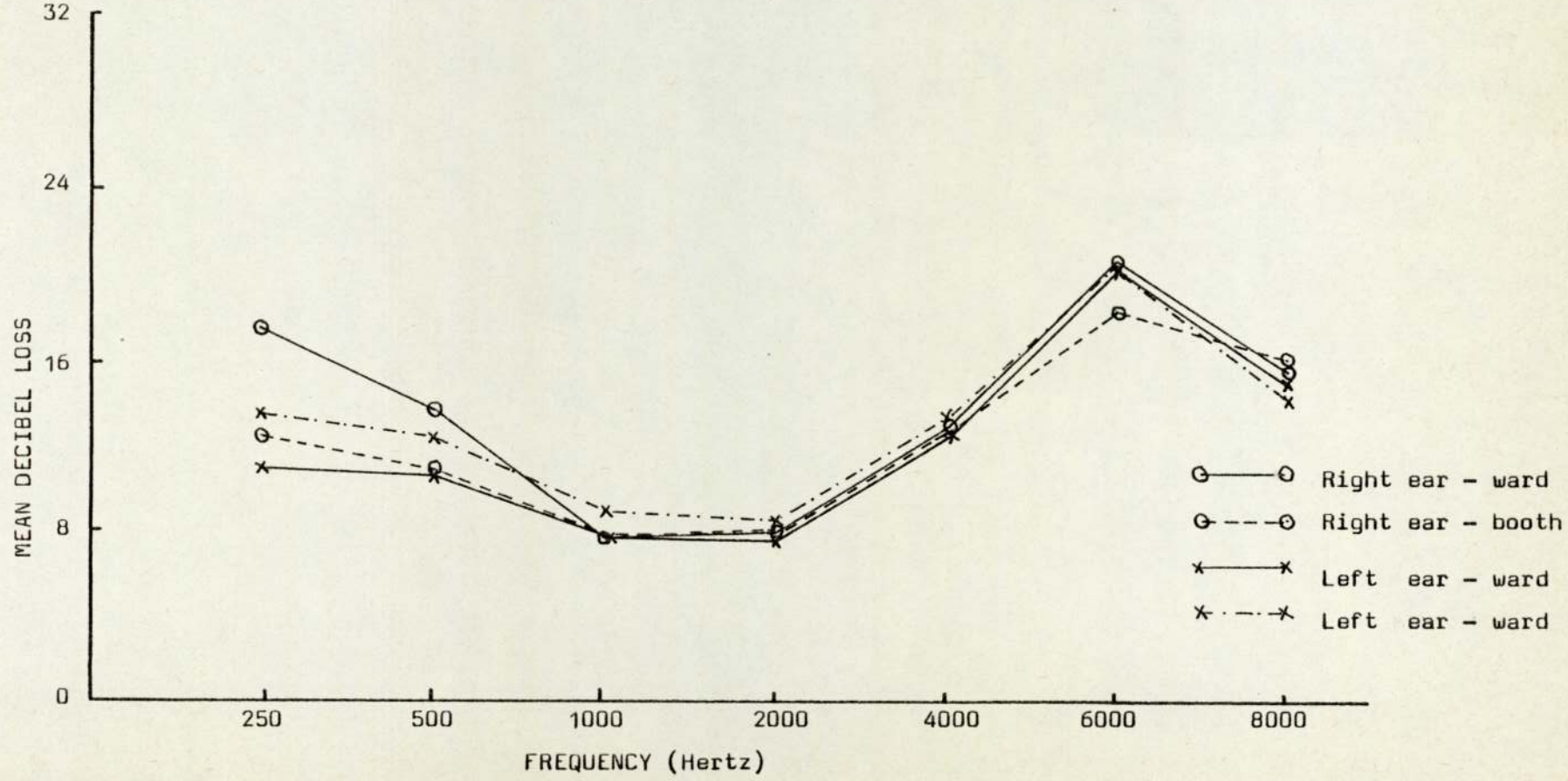


Fig. 11 Mean hearing threshold levels for the right and left ears of 24 subjects measured on the ward and in the booth using the Kamplex audiometer - bone conduction tests.

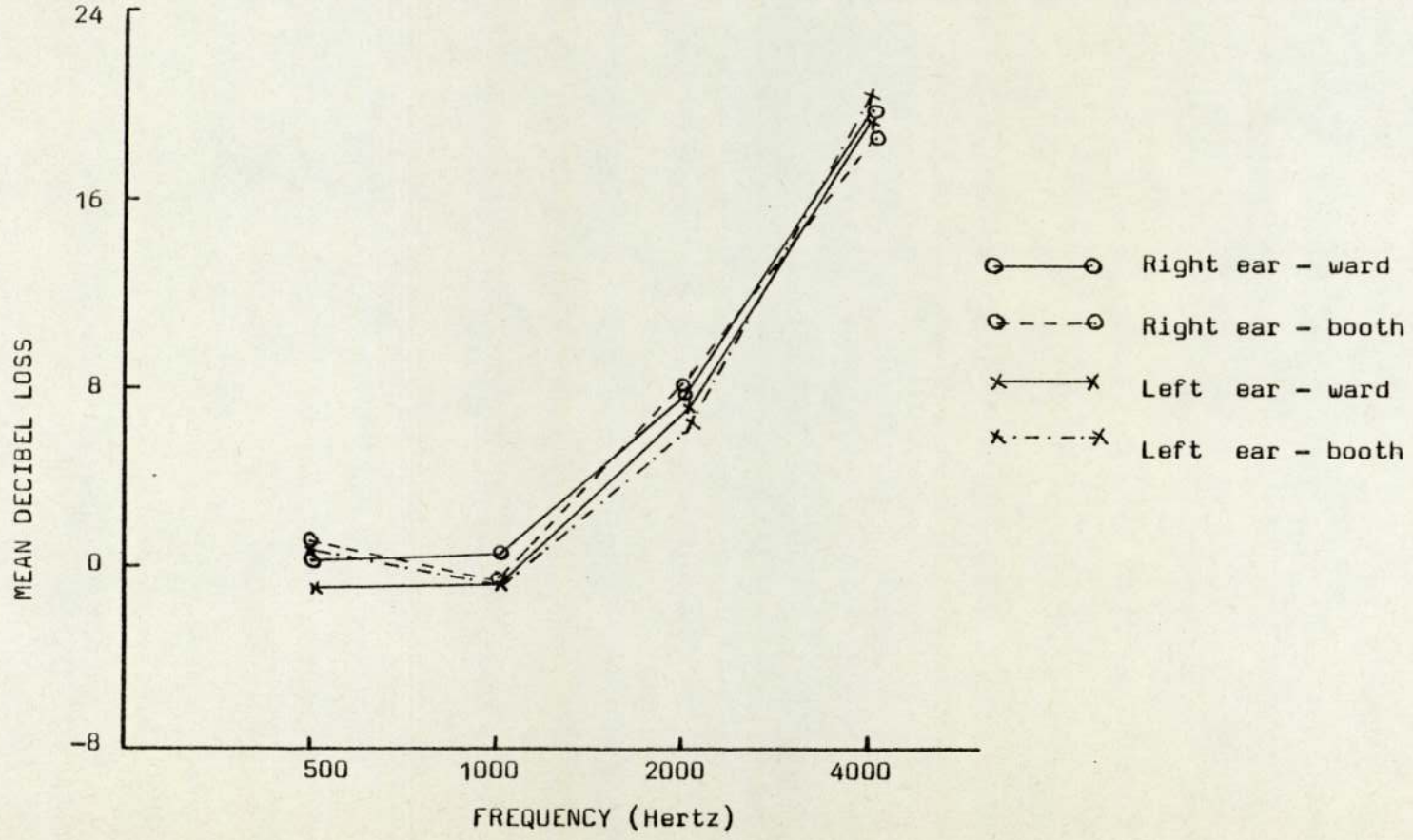


Table 13

Comparison of data obtained for the air conduction tests when tests were performed on the ward or in the sound-proofed booth

Frequency (Hz)	Ears (N=24)	Mean Value Ward	Standard Error Ward	Mean Value Booth	Standard Error Booth	t Value	*r
250	Left ear	13.5	2.8	11.0	2.2	2.63	0.64
	Right ear	17.5	3.6	12.7	2.6	4.18	0.61
500	Left ear	12.1	2.5	10.4	2.1	2.14	0.61
	Right ear	13.5	2.8	10.8	2.2	3.19	0.68
1000	Left ear	8.8	1.8	7.7	1.6	1.55	0.84
	Right ear	7.7	1.6	7.9	1.6	-0.27	0.72
2000	Left ear	8.5	1.7	7.7	1.6	1.00	0.84
	Right ear	7.9	1.6	7.9	1.6	-0.00	0.79
4000	Left ear	13.1	2.8	12.3	2.5	0.85	0.90
	Right ear	12.7	2.6	12.9	2.6	-0.18	0.78
6000	Left ear	20.4	4.2	20.4	4.2	-0.00	0.94
	Right ear	20.6	4.2	18.3	3.7	2.72	0.84
8000	Left ear	14.0	2.9	15.0	3.1	-0.96	0.89
	Right ear	15.4	3.1	16.0	3.3	-0.83	0.92

*r = correlation coefficient

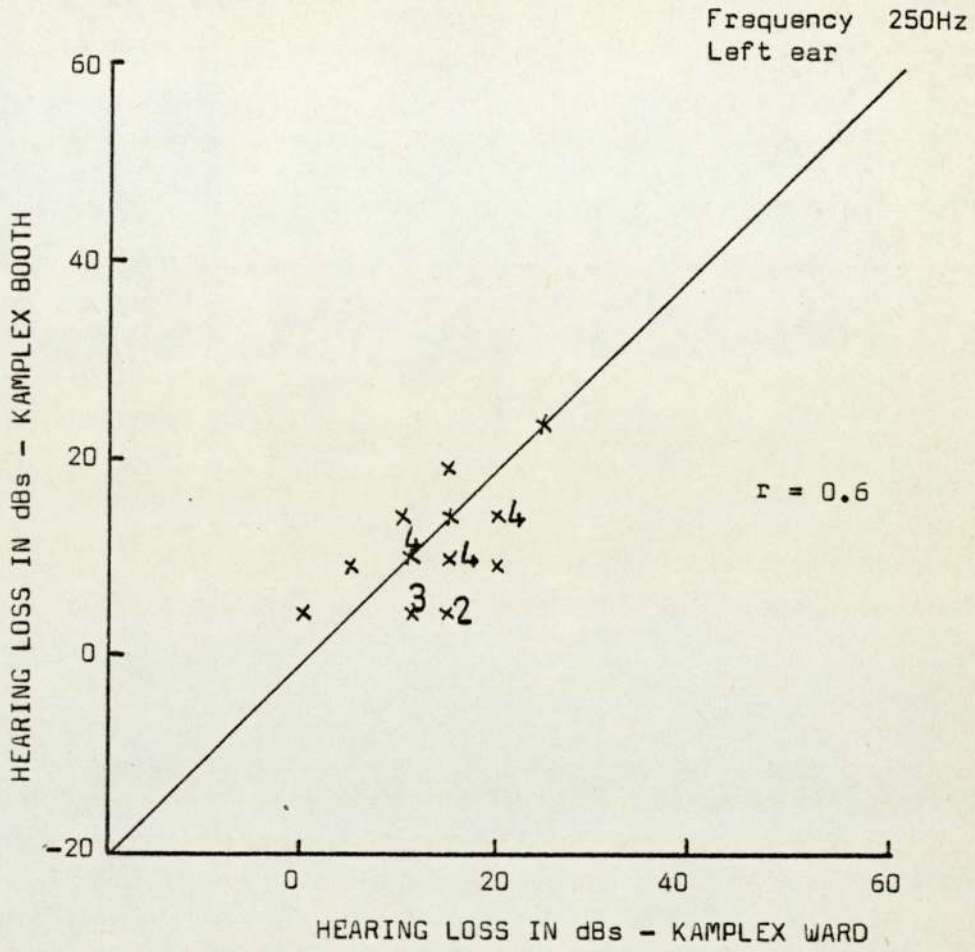
Table 14

Comparison of data obtained for the bone conduction tests when tests were performed on the ward or in the sound-proofed booth

Frequency (Hz)	Ears (N=24)	Mean Value Ward	Standard Error Ward	Mean Value Booth	Standard Error Booth	t Value	*r
500	Left ear	0.4	0.1	-1.0	0.2	1.22	0.14
	Right ear	0.2	0.0	1.0	0.2	-1.04	0.52
1000	Left ear	-0.6	0.1	-0.8	0.2	0.46	0.62
	Right ear	0.2	0.0	-0.6	0.1	0.77	0.84
2000	Left ear	5.8	1.2	7.1	1.4	-1.88	0.79
	Right ear	7.3	1.5	7.7	1.6	0.00	0.72
4000	Left ear	20.6	4.2	19.6	4.0	1.23	0.90
	Right ear	19.8	4.0	18.5	3.8	1.66	0.93

*r = correlation coefficient

Fig. 12 Comparison of the threshold levels obtained in the booth and on the ward - air conduction tests.



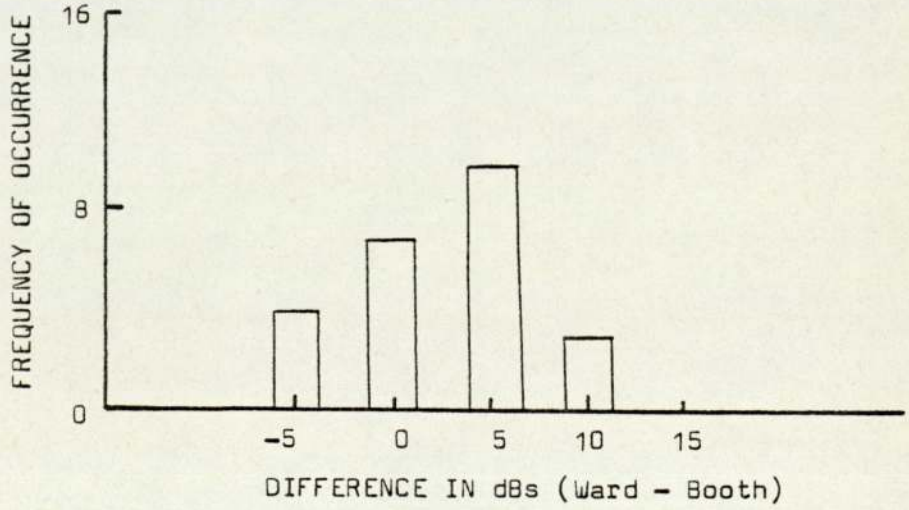
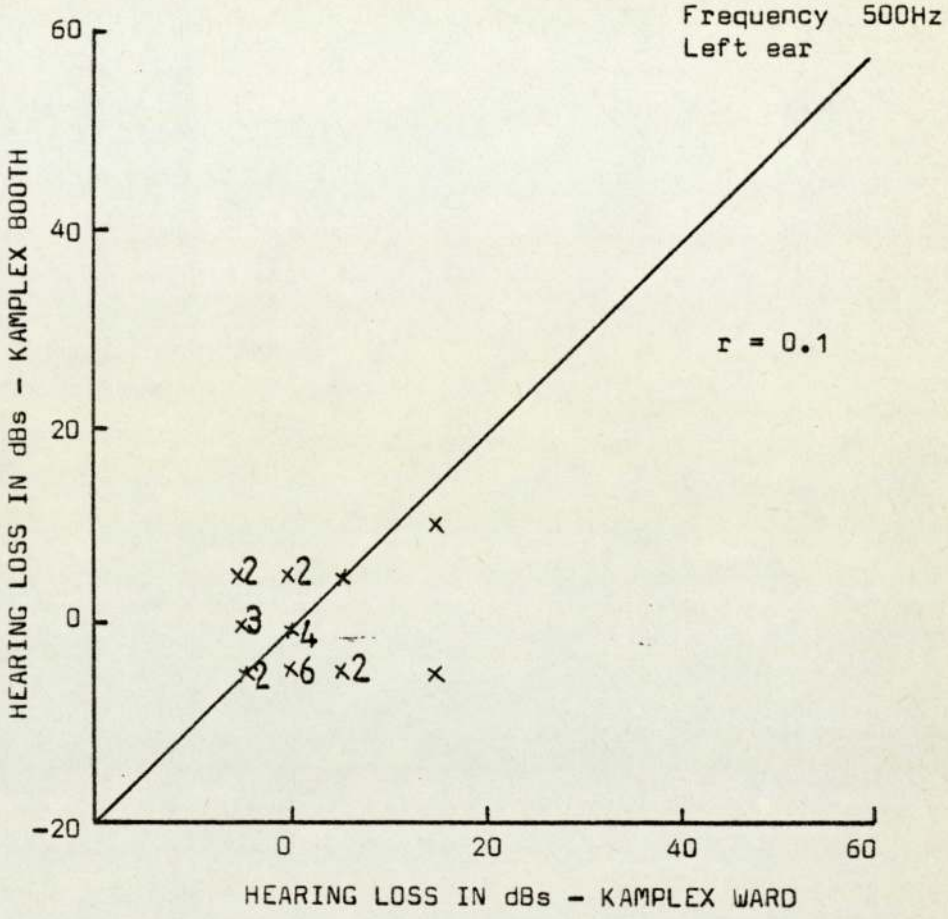
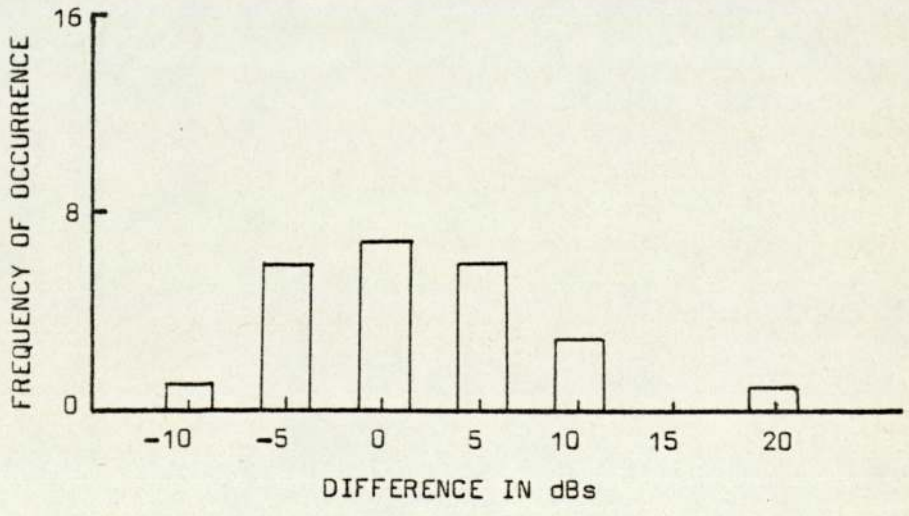


Fig. 13 Comparison of the threshold levels obtained in the booth and on the ward - bone conduction tests.





obtained in the sound-proof booth were lower than the ward values. Correlation coefficients (r) also showed a lower value at lower frequencies but the t-test showed that there was a highly significant correlation between the two sets of values. However, the bone conduction results of the t-test for the correlation coefficients showed that there was no correlation between the two sets of results for the left ear at 500Hz.

The most frequently occurring difference was 0dB at frequencies above 1000Hz for the air conduction tests and for all frequencies for the bone conduction tests. The lower frequencies, that is 250 and 500Hz, in the case of air conduction tests showed 5dB as the most frequently occurring difference.

3.5 Experiment III

Consistency of individual subjects

In this experiment the consistency of the subject himself was studied. This factor can affect the reliability of the audiometric data. The two questions that the answers were required were:

1. How reproducible were the subjects responses?
2. Was there a learning effect?

To answer these questions, subjects from Experiment II were re-used. All 24 of the subjects were re-tested approximately 24 h later on the ward using the portable Kamplex audiometer. The values obtained for the first test were compared with the second test. All the subjects, therefore, had performed three tests - two on the ward and one in the sound-proofed booth. In 50% of the cases the results compared were between test 1 and test 3 and in the other 50% of the cases, test 2 was compared with test 3. It was assumed that this would not influence the results.

The reasons for conducting the tests on the ward for this experimant were that in the clinical ototoxicity study (Section 4, Page 91), the patients could not be transported to the sound-proofed booth in the ENT Deaprtment due to their illness. Therefore, in order to investigate the consistency of these tests when they were performed on the ward with the background noise rather than the booth, the subjects were tested on the ward twice.

3.5.1 Results

The results of the two tests are shown in Tables 15 and 16. Data were subjected to statistical analyses and the various statistical factors calculated were tabulated (Tables 17 and 18). The mean values of the differences for the tests were not found to be statistically significant when the paired t-test was applied. This indicated that no improvement took place in the second attempt at any of the frequencies for either air and bone conduction tests thus concluding that learning effect had not taken place. Graphs of the mean values for both the air and bone conduction tests are shown in Figures 14 and 15.

Table 15

Data obtained from 24 subjects tested on two consecutive days - air conduction tests

	SUBJECT	AGE (yrs)	HEARING THRESHOLD LEVEL (HTL) IN DECIBELS (dB) 250Hz 1	250Hz 2	500Hz 1	500Hz 2	1000Hz 1	1000Hz 2	2000Hz 1	2000Hz 2	4000Hz 1	4000Hz 2	6000Hz 1	6000Hz 2	8000Hz 1	8000Hz 2
1	Left ear	19	15	10	10	5	10	5	10	5	0	-5	15	10	0	-5
	Right ear		10	15	10	15	5	0	5	0	5	0	10	10	15	15
2	Left ear	27	15	15	10	15	10	10	10	10	15	10	15	15	0	5
	Right ear		5	10	5	10	-5	0	5	5	20	20	20	25	25	30
3	Left ear	30	15	15	5	5	5	5	5	5	10	5	10	15	15	15
	Right ear		20	15	10	10	5	5	0	0	10	20	15	10	0	0
4	Left ear	30	15	15	20	15	25	20	20	25	10	15	30	30	30	35
	Right ear		10	10	15	10	10	10	10	10	15	15	15	15	10	15
5	Left ear	29	10	0	10	5	15	10	20	25	30	35	35	30	35	35
	Right ear		15	5	20	10	10	10	15	10	30	30	35	30	25	25
6	Left ear	26	0	0	10	5	5	5	20	15	25	25	10	15	5	5
	Right ear		0	5	10	5	10	5	15	20	20	20	25	15	5	0
7	Left ear	30	15	20	10	15	10	10	5	0	15	15	20	20	20	25
	Right ear		25	20	15	15	10	10	10	15	10	10	20	20	10	15
8	Left ear	30	15	15	10	10	5	5	5	5	15	10	15	15	15	15
	Right ear		15	15	10	15	10	10	10	15	10	15	25	20	20	25
9	Left ear	19	10	15	5	5	10	10	5	10	10	15	15	10	5	10
	Right ear		20	15	15	15	15	10	5	10	5	10	15	20	30	25
10	Left ear	29	20	25	15	20	5	10	5	10	10	10	25	20	20	15
	Right ear		25	25	20	20	5	10	0	5	5	5	25	30	20	20
11	Left ear	36	20	15	15	10	10	10	10	5	10	5	25	25	20	15
	Right ear		20	20	10	10	5	10	10	10	0	5	35	30	25	20
12	Left ear	27	5	10	15	15	5	5	5	10	10	5	10	10	5	5
	Right ear		20	15	15	20	5	10	5	5	10	15	20	20	5	5
13	Left ear	20	10	15	5	15	5	5	0	0	0	5	10	10	0	10
	Right ear		25	20	20	15	10	5	5	5	10	10	25	25	20	15
14	Left ear	22	10	10	15	15	10	10	10	10	15	20	15	15	20	15
	Right ear		20	25	10	15	5	5	0	0	10	5	5	10	0	15
15	Left ear	31	20	15	20	15	15	15	20	15	30	35	35	35	35	40
	Right ear		25	30	15	20	15	15	15	15	30	20	40	35	35	35
16	Left ear	29	20	15	10	10	5	5	0	0	5	5	15	20	10	10
	Right ear		20	25	15	10	5	5	10	10	5	10	15	15	20	25
17	Left ear	63	10	15	15	15	0	5	5	5	5	10	25	15	10	10
	Right ear		20	25	10	20	5	10	5	0	5	5	20	25	15	15
18	Left ear	28	25	25	15	20	15	15	5	10	30	25	35	30	20	25
	Right ear		25	30	30	30	20	20	20	20	30	30	25	25	25	30
19	Left ear	29	10	15	10	10	5	5	0	-5	0	5	15	10	5	10
	Right ear		15	20	15	10	0	5	5	5	5	10	15	20	10	10
20	Left ear	22	5	5	15	15	10	10	10	15	20	20	20	20	5	10
	Right ear		10	10	5	10	10	10	10	15	5	5	20	20	5	5
21	Left ear	24	20	20	20	15	10	10	10	10	20	15	30	25	15	20
	Right ear		25	20	20	15	10	5	5	0	15	15	25	20	15	15
22	Left ear	20	10	10	10	10	5	5	0	0	0	5	10	15	0	10
	Right ear		20	25	10	15	5	10	5	5	10	10	10	5	10	5
23	Left ear	24	15	15	10	10	5	10	10	5	20	20	40	35	30	25
	Right ear		15	10	10	10	5	5	10	10	25	30	15	15	15	15
24	Left ear	21	15	15	10	10	10	15	15	10	10	10	15	15	15	15
	Right ear		15	20	10	15	10	10	10	10	15	15	20	15	15	15

1 = Test 1; 2 = Test 2

Table 16

Data obtained from 24 subjects tested on two consecutive days - bone conduction tests

SUBJECT		AGE (yrs)	HEARING THRESHOLD LEVEL IN DECIBELS (dB) 500Hz 1	500Hz 2	1000Hz 1	1000Hz 2	2000Hz 1	2000Hz 2	4000Hz 1	4000Hz 2
1	Left ear	19	-5	-5	0	0	-5	0	0	5
	Right ear		0	-5	-5	-5	-10	0	10	10
2	Left ear	27	0	5	-5	0	10	10	20	15
	Right ear		0	-5	-10	-5	15	10	20	25
3	Left ear	30	0	-5	-5	-5	10	10	15	15
	Right ear		0	0	-5	-5	5	5	15	20
4	Left ear	30	5	0	5	5	10	10	20	15
	Right ear		0	5	5	5	10	10	20	20
5	Left ear	29	5	-5	-5	-5	10	5	25	25
	Right ear		-5	-5	0	-5	5	0	30	20
6	Left ear	26	0	5	10	0	20	25	30	30
	Right ear		0	5	10	5	20	20	25	25
7	Left ear	30	-5	-5	-5	0	5	10	25	25
	Right ear		0	-5	-5	-5	10	10	15	20
8	Left ear	30	0	0	-5	0	-5	-5	20	25
	Right ear		0	0	0	-5	0	5	25	25
9	Left ear	19	5	0	0	5	0	0	15	15
	Right ear		10	5	10	5	0	5	0	5
10	Left ear	29	-5	0	-5	-5	0	5	15	10
	Right ear		-5	0	-5	-10	0	-5	25	25
11	Left ear	36	-5	-5	-5	-5	0	5	15	15
	Right ear		0	0	0	5	10	10	10	10
12	Left ear	27	5	0	0	5	5	5	15	10
	Right ear		5	10	5	5	5	5	10	10
13	Left ear	20	-5	0	5	5	5	10	15	20
	Right ear		5	5	-5	-5	10	10	20	20
14	Left ear	22	15	10	5	10	10	5	20	20
	Right ear		-5	0	10	15	5	5	15	15
15	Left ear	31	0	0	0	0	5	10	30	25
	Right ear		5	0	10	5	25	25	35	35
16	Left ear	29	0	-5	0	0	10	5	25	25
	Right ear		0	-5	0	0	10	10	20	20
17	Left ear	63	-5	5	-5	5	5	5	5	10
	Right ear		-5	5	-5	0	-5	0	10	15
18	Left ear	28	10	15	5	5	5	10	35	30
	Right ear		15	10	10	5	15	20	30	30
19	Left ear	29	-5	-5	-5	-5	10	5	20	15
	Right ear		0	0	-10	-10	10	5	20	20
20	Left ear	22	0	0	5	10	10	15	25	25
	Right ear		-5	-5	0	10	10	10	15	15
21	Left ear	24	0	-5	0	-5	10	10	25	25
	Right ear		0	0	-5	-5	10	5	25	25
22	Left ear	20	-5	-5	-5	0	-5	-5	10	10
	Right ear		0	-5	-5	-5	-5	0	15	20
23	Left ear	24	0	-5	0	0	5	5	45	40
	Right ear		-5	-5	-5	0	5	10	40	45
24	Left ear	21	5	0	0	5	10	10	25	20
	Right ear		-5	0	10	5	15	20	25	25

1 = Test 1; 2= Test 2

Table 17

Comparison of data obtained for the air conduction tests when two consecutive tests were performed on the ward

Frequency (Hz)	Ears (N=24)	Mean Value Test 1	Standard Error Test 1	Mean Value Test 2	Standard Error Test 2	t Value	*r
250	Left ear	13.5	2.8	13.7	2.8	-0.25	0.77
	Right ear	17.5	3.6	17.9	3.7	-0.42	0.76
500	Left ear	12.1	2.5	11.9	2.4	0.25	0.60
	Right ear	13.5	2.8	14.2	2.9	-0.62	0.58
1000	Left ear	8.8	1.8	9.0	1.8	-0.37	0.85
	Right ear	7.7	1.6	8.1	1.7	-0.30	0.72
2000	Left ear	8.5	1.7	8.3	1.7	0.25	0.84
	Right ear	7.9	1.6	8.3	1.7	-0.62	0.85
4000	Left ear	13.0	2.7	13.3	2.7	-0.24	0.90
	Right ear	12.7	2.6	13.8	2.8	-1.23	0.88
6000	Left ear	20.4	4.2	19.2	3.9	1.54	0.91
	Right ear	20.6	4.2	20.0	4.1	0.68	0.84
8000	Left ear	14.0	2.9	15.6	3.2	-1.78	0.91
	Right ear	15.4	3.1	16.4	3.3	-1.10	0.88

*r = correlation coefficient

Table 18

Comparison of data obtained for the bone conduction tests when two consecutive tests were performed on the ward

Frequency (Hz)	Ears (N=24)	Mean Value Test 1	Standard Error Test 1	Mean Value Test 2	Standard Error Test 2	t Value	* r
500	Left ear	0.4	0.1	-0.4	0.1	0.85	0.52
	Right ear	0.2	0.0	0.2	0.0	0.00	0.59
1000	Left ear	-0.6	0.1	1.0	0.2	-2.00	0.60
	Right ear	0.2	0.0	0.0	0.0	0.24	0.79
2000	Left ear	5.8	1.2	6.9	1.4	-1.42	0.83
	Right ear	7.3	1.5	8.1	1.7	-6.40	0.86
4000	Left ear	20.6	4.2	19.6	4.0	1.42	0.93
	Right ear	19.8	4.0	20.8	4.2	-1.55	0.93

*r = correlation coefficient

Fig. 14 Mean hearing threshold levels for the right and left ears of 24 subjects measured on two consecutive days on the ward with the Kamplex audiometer - air conduction tests.

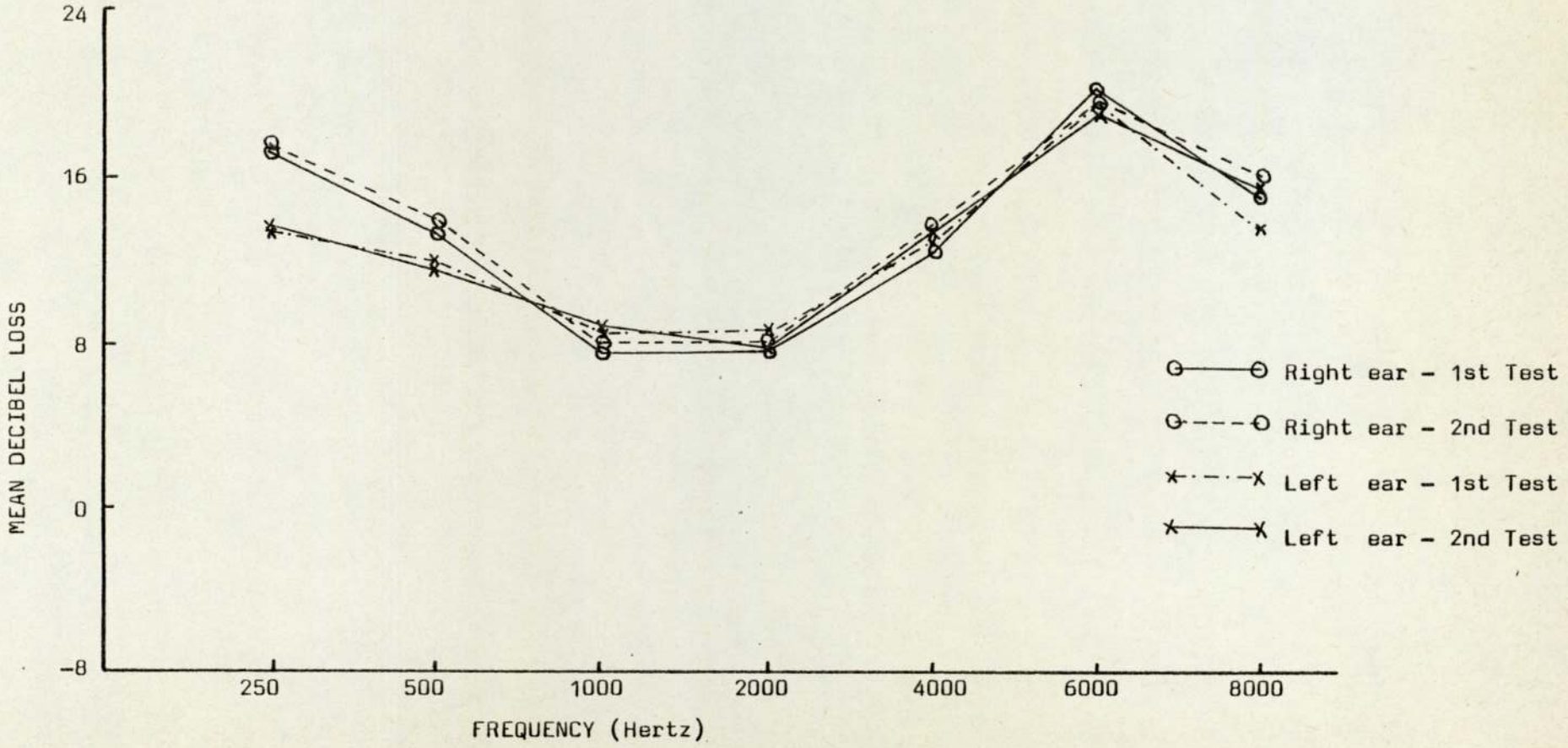
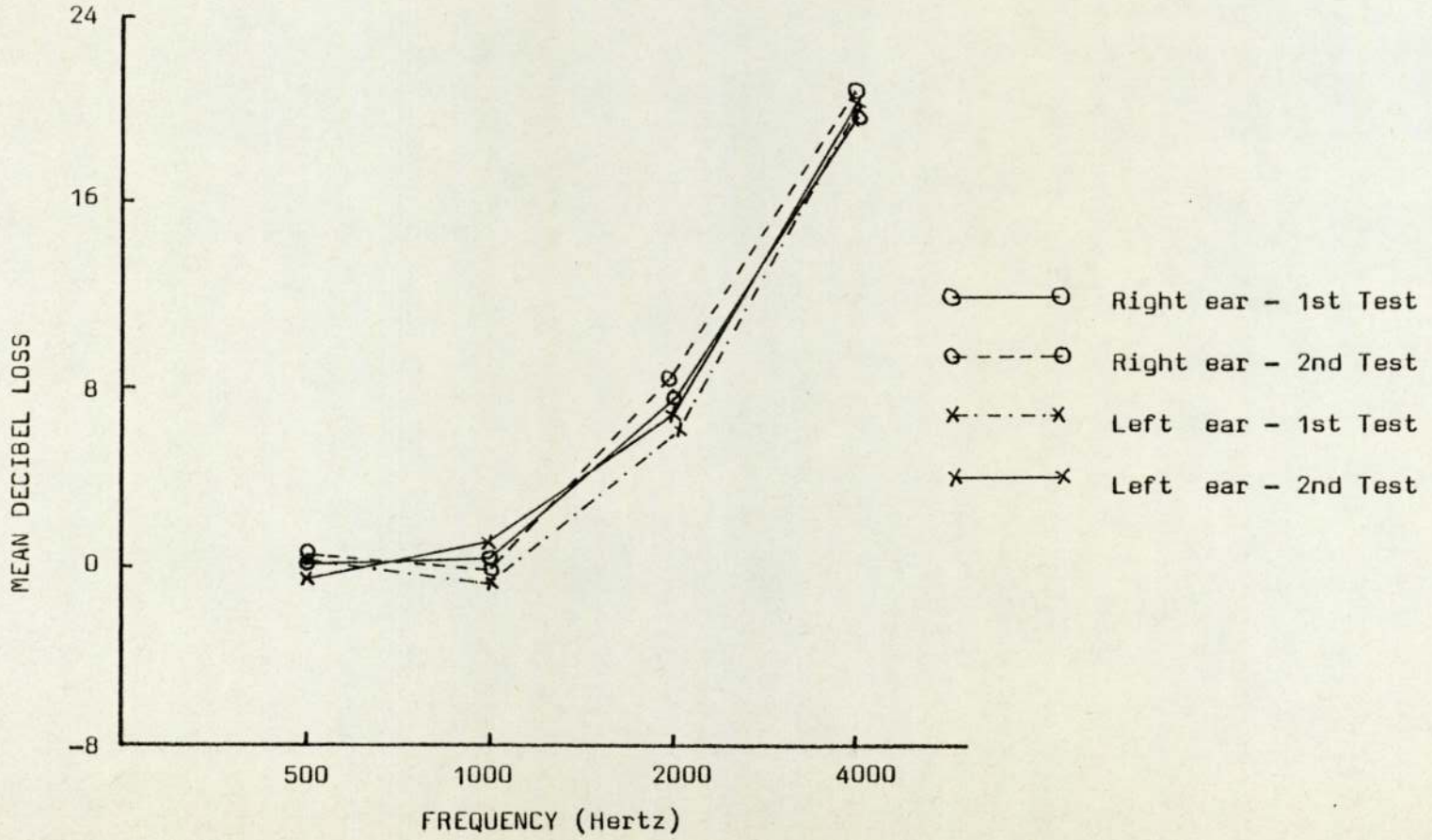


Fig. 15 Mean hearing threshold levels for the right and left ears of 24 subjects measured on two consecutive days on the ward with the Kamplex audiometer - bone conduction tests.



3.6 Discussion and Conclusions

It has been shown that, when a group of subjects had their hearing threshold levels tested first on one audiometer and then on another, the agreement between the two sets of measurements was extremely good ($P<0.001$) for both the bone and air conduction tests. Paired t-test showed that there was no significant difference between the two audiometers. The scatter of the differences relative to the mean in terms of standard deviation was less than 5dB at all frequencies for both the air and bone conduction tests.

In the second experiment, when the subjects were tested on the ward and then in the sound-proofed booth, it was found that at low frequencies ($<$ 1000Hz), there was a significant difference ($P<0.01$) between the values obtained on the ward compared with the sound-proof booth. This was not true for the bone conduction tests when it was found that there was no significant difference between the ward and the sound-proofed booth values. The differences found at low frequencies for air conduction tests administered on the ward compared to the sound-proof booth can be explained by the fact that most ambient noise is normally in the low frequency range ($<$ 500Hz).

No improvement could be found in the HTLs for the group of subjects when they were re-tested under the same conditions. This showed that the effect of one previous test on the part of the subject did not produce lower threshold values. Of course, it does not follow that in individual cases, a second test on a patient will not show a different result from that of the first, since it is quite likely to occur in practice that the patient may not be as co-operative the first time

as these normal subjects. Again difficulty arises when dealing with profoundly deaf people whose hearing is being tested for the first time and successive measurements may show improvement as the patient gets familiar with the test and starts to understand what is actually required of him. But, in general, if the test is properly conducted, it can be said that no reliable improvement takes place on the second measurement.

SECTION 4

CLINICAL OTOTOXICITY OF GENTAMICIN

4.1 Introduction

Although gentamicin is widely used today in the United Kingdom, there is no doubt that it would be used in a greater number of cases if it were not for its ototoxicity. Clinical (Dayal, Smith and McCain, 1974) and experimental (Lundquist and Wersäll, 1967) studies have clearly revealed that gentamicin possesses both forms of ototoxicity, cochlear as well as vestibular. By combining electrophysiological testing with histological methods, Hawkins and his colleagues (1969) have produced convincing evidence of early damage to the sensory epithelia in the inner ear. Waitz, Moss and Weinstein (1971) reported that gentamicin given at levels of 20, 40 and 60 mg/kg/day produced vestibular damage manifested by ataxia in cats. The length of time between initiation of treatment and appearance of ataxia was inversely related to the size of the dosage. At a dose of 60 mg/kg/day, death resulted from renal tubular damage shortly after the appearance of ataxia. At lower dosage levels, proportionally longer intervals were noted between the appearance of ataxia symptoms and death. At a dose level of 40 mg/kg/day, ataxia occurred after the same length of time whether gentamicin was given subcutaneously or intraarterially, and whether it was given in one dose or in two equally divided doses. They concluded that the peak levels of the drug were not solely responsible for the time of appearance of ataxia. Total dose and length of treatment were also considered important factors.

The vestibular damage sustained by the cats in the

Waitz et al. (1971) study appeared irreversible. Renal damage, however, proved reversible in a small number of cats after gentamicin was discontinued.

Hawkins et al. (1969) found that gentamicin administered in daily doses of 10 - 80 mg/kg produced ataxia in cats after time intervals ranging from 13 days with the largest dose to 113 days with the smallest. Post-mortem examinations revealed scarring and loss of vestibular hair cells. Cochlear damage was noted as well.

Wersäll, Lundquist and Björkroth (1969) reported that in guinea-pigs, daily doses of 100 mg/kg or more of gentamicin caused a progressive destruction of the vestibular sensory cells. Wersäll et al. (1969) reported that the cellular degeneration involved mitochondrial changes, fusion of sensory hairs and bulging of the cell surface due to 'destruction of a stable factor of the plasma membrane'. At dose levels of 40 mg/kg, damage of the hair cells appeared only after several weeks of treatment. Degeneration of the cochlear hair cells started at the basal coil of the cochlea and progressed slowly apically.

There is considerable disagreement as to the clinical incidence of gentamicin ototoxicity in patients. It is extremely difficult to assess the frequency of ototoxicity in clinical situations by reviewing the literature. Studies prior to 1970 are valueless for assessing the frequency of ototoxicity because at that time knowledge on how best to control aminoglycoside therapy was inadequate. Also, the monitoring of assays and blood levels have greatly improved since the early 1970s. Hewitt's (1974) data showed that ototoxicity

reported after the introduction of gentamicin had declined.

Survey of gentamicin VIII nerve toxicity

(from Hewitt, 1974)

Clinical investigation period	No. of case reports	No. of patients with signs or symptoms probably related to VIII nerve	Possible or probable VIII nerve toxicity
1963 - 1966	565	29 (5.1%)	16 (2.8%)
1967 - 1969	1450	42 (2.8%)	27 (1.8%)
1970 - 1973	1635	17 (1.0%)	15 (1.0%)

These figures show a decline from 2.8% to 1.0%. The other problem related to comparisons of ototoxicity is that ototoxicity is multi-factorial, which increases the difficulty in assessing studies. The important risk factors have been reported to be:
renal impairment (Jackson and Arcieri, 1971);
long duration of treatment (Finegold, 1966);
high total dose (Fee, Vierra and Lanthrop, 1978);
previous or concomitant ototoxic drugs (Finegold, 1966);
elevated serum levels (Nordström et al., 1973); and
increased age (Finegold, 1966).

Many of these risk factors are interlinked. For instance, long duration of treatment goes with high total dose. Renal impairment goes with high levels and so on. This inter-relationship was illustrated in a study by Black and his colleagues (1976) using amikacin. With five risk factors, three out of three patients had ototoxicity which was confirmed audiometrically. With four risk factors, six out of six patients were affected. However, as the number of risk factors was reduced, so did the incidence of the hearing loss. They concluded that the

risk of ototoxicity increased markedly when three or more risk factors were present.

In a retrospective study undertaken by Banck et al. (1973), electronystagmographic (ENG) testing was performed in 35 patients previously treated with gentamicin. Seven of these patients showed definite abnormalities and had severe bilateral vestibular dysfunction. Six of the seven had had symptoms during or after treatment and in these vestibular damage was probably due to gentamicin. Hearing was impaired in all patients with vestibular damage, but only one patient had noticed such impairment developing during treatment. In six of the patients, ENG was repeated after six to twelve months; only in one was any significant vestibular improvement observed.

In order to evaluate the risk of ototoxicity during gentamicin therapy, a prospective study was undertaken (Nordström et al., 1973). Otoneurological examination with audiometry and ENG with caloric stimulation in 34 patients was performed. Six of the 34 pateints had severe ENG abnormalities after treatment. In three patients, the connection between the pathological findings and the administration of gentamicin was regarded as certain. Thus the incidence of vestibular ototoxicity was 18% of which it was considered that gentamicin was probably responsible for changes in 10%. In their study, the patients treated with gentamicin were generally elderly and often had impaired renal function. These factors were thought to have contributed to the high frequency of ototoxicity in their study. Nordström et al. (1973) studied eight potential risk factors and found significant correlation between vestibular

dysfunction and three of these potential risk factors: duration of treatment, serum creatinine level and gentamicin trough level (amount of drug present prior to the next dose).

In a comprehensive, retrospective study, Jackson and Arcieri (1971) reviewed 1484 case summaries of patients treated with gentamicin during the period 1966 to 1969 throughout the U.S.A. 42 courses were accompanied by symptoms and/or signs that suggested vestibular or auditory dysfunction. Drug-associated ototoxicity for this group was rated as 2.8%. On critical analysis, however, one-third of the toxic effects reported were thought to be unrelated to gentamicin making the true incidence for the four year period to be approximately 2%. This study further showed that in approximately two-thirds of the patients with ototoxic effects, dysfunction was limited to the vestibular system and one-third to the auditory system. Symptoms of ototoxicity usually appeared between the first and second week of treatment but were observed as early as the third day in a few instances. In one patient, symptoms were unrecognized until the ninetieth day of treatment.

Effects were transient in more than one-half of the patients with symptoms of labyrinthine dysfunction. In others there was no evidence of recovery. Hearing losses were mild and restricted to high frequencies.

Jackson and Arcieri (1971) concluded that the most critical factors in development of ototoxicity were impaired renal function and the size of the daily dose.

Other studies conducted by several workers have also substantiated the fact that gentamicin is both cochleo- and vestibulotoxic (Dayal, Whitehead and Smith, 1975; Dayal, Smith

and McCain, 1974). Fee, Vierra and Lathrop (1978) showed that 20% of the 45 patients studied demonstrated ototoxicity. Pure-tone and speech audiograms together with caloric testing using ENG were performed prior to or within 48 h of the start of drug administration and periodically thereafter. Four patients had a hearing loss while another four had vestibular dysfunction. One patient demonstrated both cochlear and vestibular dysfunction. Most of the cases of cochlear toxicity were not reversible and resulted in apparent permanent loss of hearing mostly at frequencies above 4000Hz. It was surprising that patients who developed cochlear toxicity had a statistically significant higher mean creatinine clearance on day 3 than those who did not become toxic. Bender, Fortner, Schimpff, Grove, Hahn, Love and Wlernik (1979) studied 32 patients and found gentamicin-induced ototoxicity in only two patients (6.4%).

From these and other studies (Finegold, 1966) it is clear that the earliest sign of auditory damage in cases of aminoglycoside antibiotics is usually tinnitus, and although ototoxic effects may occur in the absence of tinnitus, its presence in presumptive evidence for the onset of cochlear toxicity. Hearing loss is invariably recruiting (an abnormal rapid growth of loudness) sensorineural (defects are either in the cochlea itself or in the fibres of the auditory nerve: therefore, threshold elevations observed in both the air and bone conduction tests) with either unilateral or bilateral involvement (Dayal, Whitehead and Smith, 1975) although the latter is more often observed. Upon audiometric evaluation, high frequency losses are more often present than losses in the low frequencies. Though important, early detection may

be difficult. Since it is well recognized that humans can hear frequencies up to approximately 20,000Hz but most audiometers test only as high as 8000Hz, one can readily see how damage can be present but remain undetected despite regular audiometric monitoring. Further complication in diagnosis results from the fact that the patient may not notice or complain of a hearing loss until it exceeds approximately 30dB and encroaches on the frequencies below 2000Hz. Although the hearing loss that occurs has on occasions been noted to be reversible (Black, Lau, Weinstein, Young and Hewitt, 1976), it is most often permanent. The earlier the loss is noted, the better appears to be prognosis for hearing impairment if therapy is discontinued.

The first vestibular symproms observed are tinnitus and ataxia or vertigo. Damage is usually permanent and affected patients remain insecure on ambulation, particularly in the dark, but they can eventually compensate for the disability (Jao and Jackson, 1964).

A number of audiometric tests could theoretically be applied apart from the pure-tone air and bone conduction tests to determine this cochlear loss. These tests are:

Speech discrimination

This test consists of evaluating the individual's ability to repeat correctly a list of phonetically balanced monosyllabic words presented at comfortable loudness levels. Speech discrimination scores are low with lesions involving the cochlea (Egan, 1948).

In the early stages of the drug-induced loss, when the loss is confined to frequencies outside the critical speech

range, speech discrimination scores may not be seriously affected. The patient may be aware merely of a degree of frustration while trying to communicate under difficult listening conditions. However, as the loss extends to the speech frequencies, communication can become difficult.

Tone decay

This phenomenon whereby a patient exhibits a progressive elevation (decreased sensitivity) of this auditory threshold for a sustained test tone as a function of time. Usually no tone decay is observed but with a severe cochlear involvement the 4000Hz frequency may demonstrate significantly more tone decay than normals (Hood, 1956).

Bekesy audiometry

With Bekesy audiometry, the patient records his own audiogram by depressing and releasing a signal key. A sawtooth-shaped curve, that sweeps across the frequency range (250 - 8000Hz), is plotted. Each ascending arm of the Bekesy trace represents a period of audibility while the descending arm indicates a period of inaudibility.

When audiograms are obtained for periodically interrupted (I) and continuous (C) tones, a type II tracing which is characteristic of cochlear pathology is observed (Jerger, 1960). With this the C threshold separates from I at around 1000Hz and travels parallel to the high frequency end of the audiogram. The separation usually does not exceed 20dB. In addition, the amplitude of the continuous tone tracing narrows considerably to 3 to 5dB.

Loudness recruitment

Patients with hearing losses due to certain cochlear

lesions characteristically exhibit a growth in the sensation of loudness with increasing stimulus intensity that is more rapid than in normal subjects. This phenomenon is termed loudness recruitment and is accounted for by the loss of the sensory elements which respond to the weakest stimuli (Evans, 1975).

The simplest test for loudness recruitment is the alternate binaural loudness balance (ABLB) test which can be performed when there is a substantial difference (at least 20dB) between the threshold intensity levels of the two ears at the same frequency. In this test, a record is made of the intensity levels required in the normal ear to produce equal loudness sensation to a range of sensation levels in the impaired ear (Priede and Coles, 1974).

Initially, when this present study was undertaken, it was anticipated that each gentamicin-treated patient would undergo if not all, some of the above mentioned tests at regular intervals but it soon became apparent that most of our patients were too ill to co-operate with all these tests. It was not also possible to transport them to the ENT clinic where the equipment required to perform these tests was based. In order to perform all these tests, one would have to spend at least 4 h testing each patient.

It may be possible to apply objective tests to detect these hearing losses. One such test which has been applied successfully (Wilson and Ramsden, 1977) is electrocochleography (ECochG) which records acoustically evoked electrical potentials. It is particularly useful in patients who are not co-operative or patients with communication problems. Results obtained by ECochG are

highly reproducible and are unaffected by anaesthesia or unconciousness (Yoshie, 1973). However, this technique of measuring hearing loss is relatively costly, when one considers the medical and scientific staff required, let alone the equipment necessary. Other disadvantages are:

1. Anaesthesia - either local or general is required.
2. Some medical skill is required to place the electrode through the tympanic membrane onto the cochlear promontory.
3. Trauma resulting from electrode placement. This is invariably minimal but one case of middle ear infection has been reported (Schmidt and Spoor, 1974).

To detect vestibular ototoxicity, the bithermal binaural caloric test as described by Fitzgerald and Hallpike (1942) is the test which is normally used. Obviously, many patients receiving gentamicin are too ill to undergo such time consuming assessment of the vestibular system. The cold caloric test using 20 ml of water at 20^{o}C was applied on some patients but this test was found to give variable results when successive tests were performed on the same patient. This could probably be explained by the fact that when a small amount of water is used, the time of injection into one ear, contrasted to the other, presents a variable which probably has a bearing on the end-result. Using this stimulus resulted in a feeling of nausea, pallor and sweating in some patients which can be uncomfortable. Also the cold stimulus can sometimes arouse a dormant spontaneous nystagmus either by irritation of the wall of the outer ear canal or by causing psychological stress. The direction of this nystagmus is always in the direction of the stimulated

ear (Jongkees, 1973).

On reviewing the literature relating to monothermal testing, it was found that a number of workers (Bernstein, 1965; Hart, 1965; Hichcliffe, 1967; Dayal and Farkashidy and Kuzin, 1973) had expressed a preference for hot irrigation caloric tests. Before applying it on patients, the limits of this test were evaluated using 25 normal subjects. The results of this study are reported in Section 2, Page 19 . This test was found to be unreproducible when applied under non-ideal conditions using portable equipment. Due to this variation, it was therefore decided that by applying this test on patients, unnecessary discomfort would be caused without obtaining any meaningful results.

In this study, however, the vestibular symptoms were detected by applying simple co-ordination and gait tests whenever possible. These were:

1. Romberg test

   This test evaluates truncal equilibrium and is performed by asking the patient to stand with heels and toes together, arms at his side and his eyes open. He is then asked to close his eyes and the examiner observes whether or not the patient is able to maintain this position without falling or moving (Graybiel and Fregly, 1966). With labyrinthine disease, the patient falls to one side consistently.

2. Past pointing

   This is a vestibulospinal test of the upper extremities. Smooth performance requires integration of the vestibular, ocular, proprioceptive cerebellar systems.

   In this test the patient extends his arms and places

his extended index fingers on the examiner's fingers. The patient then closes his eyes and raises his arms over his head while keeping the elbows straight. He then slowly lowers his arms to return the index fingers to the starting point (Nyman, 1945).

3. Finger-to-nose test

This starts with the patient's finger on the examiner's finger. The patient then moves his index finger from the examiner's finger to nose and from his nose to the examiner's finger. The examiner, however, moves his finger to different points in space (Busis, 1969). Any lack of co-ordination suggests cerebellar or labyrinthine disease.

Vestibular symptoms such as vertigo, dizziness or imbalance were noted if they appeared in any patient.

This study was conducted to detect the prospective ototoxicity of gentamicin. Auditory toxicity was monitored by performing pure-tone air and bone conduction tests at regular intervals. Vestibular toxicity was determined by the appearance of symptoms such as vertigo or dizziness. Maintenance of balance and co-ordination of extremities was observed by performing the above tests.

A study conducted at the hospital (Talbot, 1978) showed that from January to June, 1978, tobramycin was prescribed three times and kanamycin and amikacin only once. However, gentamicin (16%) was precribed regularly and was mostly used in general surgical wards. Gentamicin was therefore singled out for this clinical study.

At the commencement of this study, the collaboration of ward pharmacists and clinicians in charge of the surgical

and renal wards was enlisted. They were given a protocol which required them to report all patients on their specific wards prescribed aminoglycosides. Unfortunately, the response to this was not good. Occasionally, when they did report a patient he had already had a number of doses of the drug or the patient was too ill to co-operate with the audiometric tests. Therefore, patients were generally obtained by going around individual wards and checking through the patients drug reports. This was obviously a tedious and time-consuming matter and sometimes some patients were missed. This is reflected in the small number of patients studied. Another problem in obtaining patients was that during this study clinical trials on some cephlosporins began so, most of the doctors were prescribing these rather than the aminoglycosides which normally did.

4.2 Methods and Materials

4.2.1 Patient selection

Between September 1977 and May 1980, gentamicin-related ototoxicity was studied at East Birmingham Hospital in a group of 35 patients of both sexes (23 male, 12 female) suffering from various diseases (Table 19). The criteria used for inclusion in this prospective study were that:

1. The minimum period of treatment with gentamicin was likely to be 5 days.
2. Patients were able to understand the instructions and were able to co-operate reasonably with audiometric testing.

Due to the nature and severity of their illnesses it was not possible to transport these patients to the ENT Department for audiometric testing in the sound-proofed booth so a portable audiometer was used to perform these tests on the ward. As far as possible all tests were performed at about the same time each day on every patient.

4.2.2 Audiometric changes in a control group of hospitalized patients

It is conceivable that due to the severity of the underlying disease, patients may not be able to co-operate very well with audiometric testing. Also, there is a possibility that unknown incidence of hearing loss in patients, attributable to factors other than gentamicin, may occur. Since it had been shown at East Birmingham Hospital that most courses of gentamicin were started post-operatively in the surgical wards (Talbot, 1978), it was decided that all patients admitted for surgery would be tested in the same manner as

the gentamicin-treated patients but before surgery and then at regular intervals for the period of their stay in hospital. However, in this case, baseline audiograms were obtained from each patient at the time when they were able to co-operate well with audiometry. If any of these patients were then started on gentamicin, they were then eliminated from the control group and transferred in the gentamicin-treated group. It turned out that only one patient was started on gentamicin from this group. However, the patients in this group were tested in the same manner as the gentamicin-treated patients and the responses obtained from this control group of patients were compared with the gentamicin-treated patients. 25 patients (17 male, 8 female) which were well-matched for age and the respective infections with gentamicin-treated group were studied (Table 19).

4.2.3. Determination of serum levels and renal evaluation

11 of the 35 gentamicin-treated patients were initially started on a fixed dose of gentamicin (80 mg every 8 h) by the clinicians in charge. In 4 of these patients, the dose was subsequently reduced as determined by their body weight and renal function. Of the 35 gentamicin-treated patients studied audiometrically, 25 (71%) were included in a concomitant pharmacokinetic and nephrotoxicity study which was carried out by Dr. P. Davey. For assays of gentamicin in serum, 10 blood samples were drawn. 5 of these samples were drawn at 0, 15, 30, 45 and 60 minutes while the other 5 were drawn at the time intervals between 2 to 8 h. Time-concentration curves were constructed from which the elimination rate, the apparent volume of distribution, area under the curve

Table 19

Classification of the control and gentamicin-treated patients suffering from various diseases

	Number of patients	
	Gentamicin-treated	Control
Post operative infections	12	12
Pyrexia in neutropenic cancer or leukaemia	7	6
Septicaemia or serious infections	6	1
Mild infections	10	6
TOTAL	35	25

and the half-life were calculated. The two kinetic parameters, elimination rate and the apparent volume of distribution were used to calculate the dose in these 25 patients and doses were altered if blood levels were high. Doses were checked by taking blood levels throughout treatment and by repeating the estimation of the elimination rate and volume of distribution by using the 5 blood levels taken at times 0 - 8 h. A Fortran computer programme was developed which was used for calculations of pharmacokinetic parameters and for plotting of graphs on data obtained from each patient.

Renal function was evaluated in 25 gentamicin-treated patients before and during gentamicin treatment at least twice a week by serum creatinine, blood urea nitrogen (BUN), creatinine clearances and urinary electrolytes. 24 h urine collections were collected everyday to study the sensitive nephrotoxic parameters which involved the studying of low molecular proteinuria and enzymuria. β_2-microglobulin was the protein measured while alanine aminopeptidase and N-acetylglucosaminase were the two enzymes studied. It is too early to say whether these sensitive measures have any advantage over the conventional methods. β_2-microglobulin and the two enzymes were not found to increase significantly before serum creatinine in the detection of nephrotoxicity.

During the second day of therapy, gentamicin serum concentrations were determined by a bioassay method using Klebsiella NCTC 10986 as an indicator organism. Serum concentrations were measured at 'zero time', i.e. just before the next dose (trough value) and the peak concentrations were determined at 30 min (intravenous injection) or at

1 h (intramuscular injection).

Subsequent doses were adjusted in the light of serum concentrations and renal function. Subsequently, the serum concentrations were determined every third day.

Of the 35 gentamicin-treated patients studied, the route of gentamicin administration was:
In 18 (51.5%) patients , an intravenous injection of gentamicin infused over a period of 5 min was given;
6 (17%) of the patients were given an intramuscular injection; and 11 (31.5%) patients were given both intramuscular and intravenous injections.

4.2.4 Test procedure

The reasons for performing the tests were explained to each patient and their informed consent obtained. A brief history including occupational exposure to noise, pathologies involving the ears, current hearing deficits or renal impairment, previous treatment with aminoglycosides or other ototoxic drugs, history of military service and a family history of hearing impairment was obtained.

The audiograms were then recorded in the manner previously described (Section 3; Page 45) and the condition of patient at the time of audiogram was noted. Pure-tone air conduction and bone conduction tests were performed. In the gentamicin-treated patients, the maximum acceptable time interval from the first dose of gentamicin for an initial audiogram was 72 h. A baseline audiogram was defined as an audiogram obtained before gentamicin therapy and the initial audiogram was defined as an audiogram obtained within 72 h of the start of therapy. In the control patients, the baseline audiogram

was obtained before surgery. Serial audiograms were then recorded every two or three days until discharge, for both the gentamicin-treated and the control patients, in order to document the onset, progression, stability or regression of any hearing deficit. Follow-up audiograms were obtained at least six weeks after the last dose in the gentamicin-treated patients and in the control group the follow-up audiogram was obtained at least six weeks after surgery. These follow-up audiograms were performed in the sound-proofed booth using the Amplivox audiometer in the ENT Department. The results of all the serial audiograms were then compared with the baseline or the initial audiograms.

The patients' case notes and results of audiometric and vestibular function tests from the gentamicin-treated patients were reviewed to determine:

1. Whether the patient had incurred any inner ear effects;
2. Whether the effects were auditory, vestibular or both; and
3. Whether the effects were related to the drug.

Initially the audiometric losses demonstrated by the gentamicin-treated group were compared with the losses demonstrated by the control group patients. They were considered to be drug-related only if the losses did not coincide with the severity of their illness or any other obvious cause. If the effects were drug-related, risk factors often associated with aminoglycoside ototoxicity (Jackson and Arcieri, 1971; Nordström et al., 1973) were examined. These were: advanced age; renal impairment; prior or concomitant therapy with other aminoglycosides or other drugs which might be ototoxic; length of treatment;

total dose and exposure to noise.

Audiometric loss was defined as an increase of at least 20dB in hearing threshold at any frequency in either ear detected by audiometry. Vestibular disturbance was defined as any symptom reported by the patient (e.g. vertigo or dizziness) or the presence of ataxia or nystagmus detected by direct observation by the examiner.

4.2.5 Classification of hearing loss

All audiograms were corrected for the hearing losses existing due to age (Glorig and Davis, 1961) using the values listed in Table 20. This was to assess whether any hearing losses were present in any of the gentamicin-treated as well as the control patients.

The following classification of hearing losses was used:

20 - 25dB	Not significant
26 - 40dB	Mild hearing loss
41 - 55dB	Moderate hearing loss
56 - 70dB	Marked hearing loss
71 - 90dB	Severe hearing loss
91 plus	Profound hearing loss

4.2.6 Computer plots

A computer programme (Appendix I) was developed which was used to plot the results of all the audiometric data obtained from each patient. The program MINIG2 was written in Fortran using GINOF subroutines on an ICL 19045 computer. The computer plotted out resultant audiograms of the differences obtained when the serial audiograms were compared with the baseline or the initial audiograms. An example of the graph obtained from the data of one patient is shown in

Table 20

Correction of the audiometric baseline or initial audiograms according to age

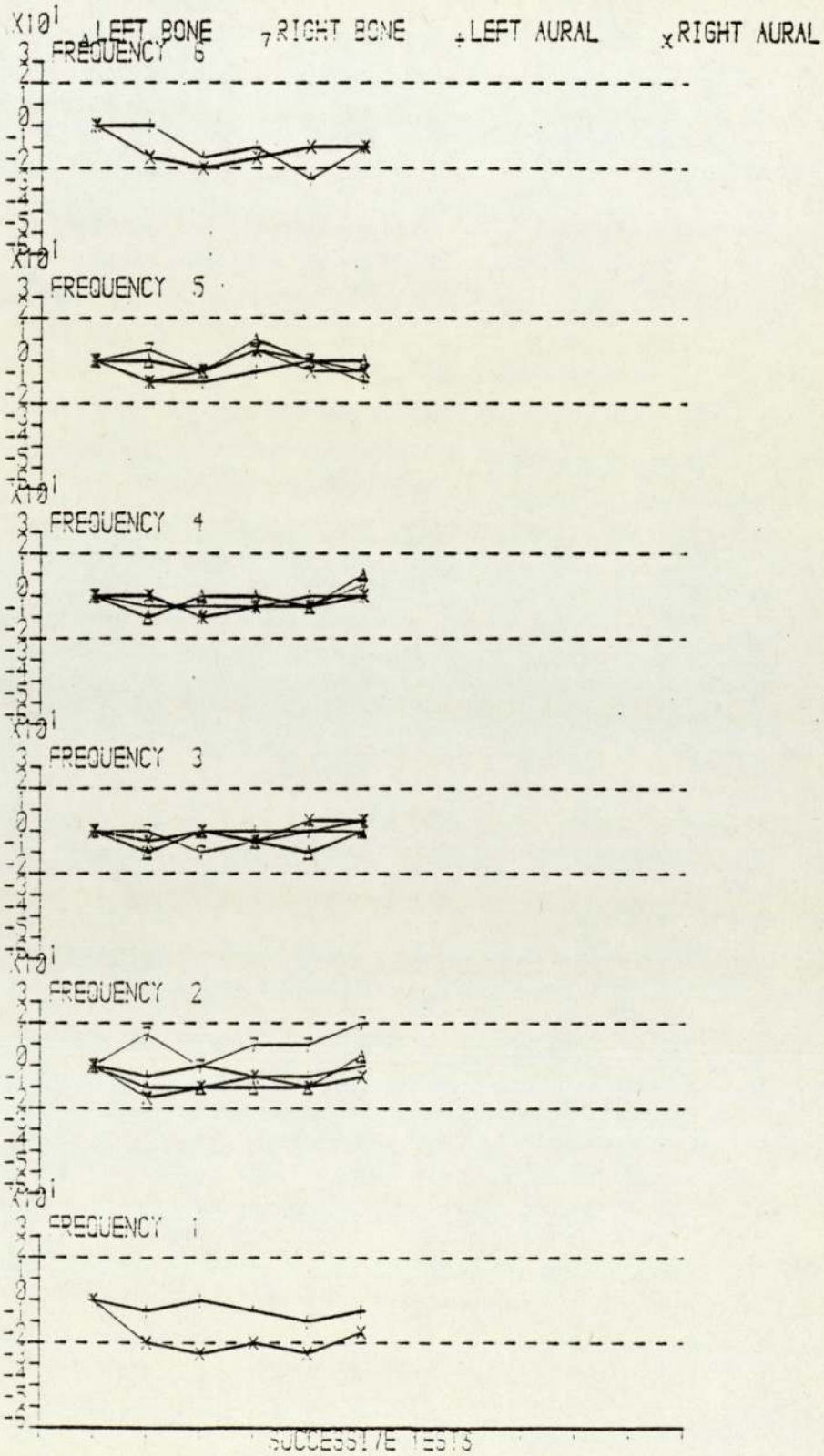
Mean 'physiological' hearing loss in decibels (dB)

Age in years	Frequency in Hertz (HZ) 250	500	1000	2000	4000	6000	8000
Males							
20 - 24	0	0	0	0	0	0	5
25 - 29	0	0	0	0	5	10	10
30 - 34	0	0	0	5	15	15	15
35 - 39	0	5	5	5	20	20	20
40 - 44	5	5	5	5	20	25	25
45 - 49	5	5	5	5	25	30	30
50 - 54	10	10	10	10	30	35	35
55 - 59	10	10	10	15	35	40	40
60 - 64	10	10	15	20	40	45	45
65 - 69	15	15	20	25	45	50	50
70 - 74	15	20	25	30	50	55	55
75 - 79	15	20	25	35	55	60	60
80 ———	25	25	30	40	60	65	65
Females							
30 - 34	0	0	0	0	5	5	5
35 - 39	5	5	5	5	10	10	10
40 - 44	5	5	5	5	15	15	15
45 - 49	5	5	10	10	15	20	20
50 - 54	10	10	10	10	20	25	25
55 - 59	10	15	15	15	25	30	30
60 - 64	15	15	20	20	30	35	35
65 - 69	15	20	20	25	35	40	40
70 - 79	20	25	25	30	40	45	50
80 ———	25	30	35	35	45	55	55

Figure 16. From these computer plots, any patients demonstrating changes greater than 20dB at any frequency for either the bone or air conduction tests, during the period of testing could be selected out.

This method was not found to be particularly useful as it was found to be more time consuming than plotting the resultant audiograms by hand. Also limits of 20dB were set for the computer to detect, and if any patient was found to be above these limits, then it was necessary to refer to the original audiograms to determine the actual hearing loss.

Fig. 16 Computer plot of the data obtained from a gentamicin-treated patient demonstrating the changes of hearing threshold levels.



4.3 Results

4.3.1 Patient control group

In this control group, only 7 of 25 (28%) patients presented for the follow-up test. 18 (72%) of them were asked at least twice to attend for a follow-up test, but there was no response. 5 (20%) control patients demonstrated audiometric losses (Table 21). In three patients, fluctuating hearing losses or gains were recorded. These losses reversed within two days and were probably related to severity of their illness at the time of the tests because they coincided at the time when they were noted to be unco-operative. However, 2 (8%) patients demonstrated permanent unilateral losses.

Case 1: This 68 year old man was admitted for cholecystectomy and at admission a marked bilateral sensorineural hearing loss was recorded. He was well enough at the time of admission and gave his full co-operation at the time of the tests. One day after the operation, the patient was febrile and was started on a course of cephazolin. Two days later, he demonstrated audiometric losses of 20dB in the right ear at frequencies 2000, 6000 and 8000Hz and these were sustained at the time of discharge.

Case 2: This 55 year old patient was also admitted for cholecystectomy and at admission had normal hearing when tested. One day after the operation, the patient developed a staphylococcal wound infection and was started on a seven day course of ampicillin. Two days after the operation, he showed a mild sensorineural hearing loss in the left ear at frequencies 4000, 6000 and 8000Hz. 11 days after the operation he was started on flucloxacillin because the

Table 21

Auditory changes in the control and the gentamicin-treated patients

	Numbers of patients	
	Gentamicin-treated	Control
Symptomatic hearing loss	2	0
Hearing losses greater than 20dB at any frequency detected during treatment and persisting at the last audiogram	2	2
Hearing losses greater than 20dB at any frequency detected during treatment and reversing at the last audiogram	1	0
Hearing losses and subsequent gains greater than 20dB at any frequency	6	3
*Hearing gains greater than 20dB at any frequency	4	2

*In these patients hearing apparently improved after the baseline/initial audiogram

infective organism was resistant to ampicillin. The hearing loss had not improved at the time of discharge or at the follow-up test which was conducted six months later (Fig. 17).

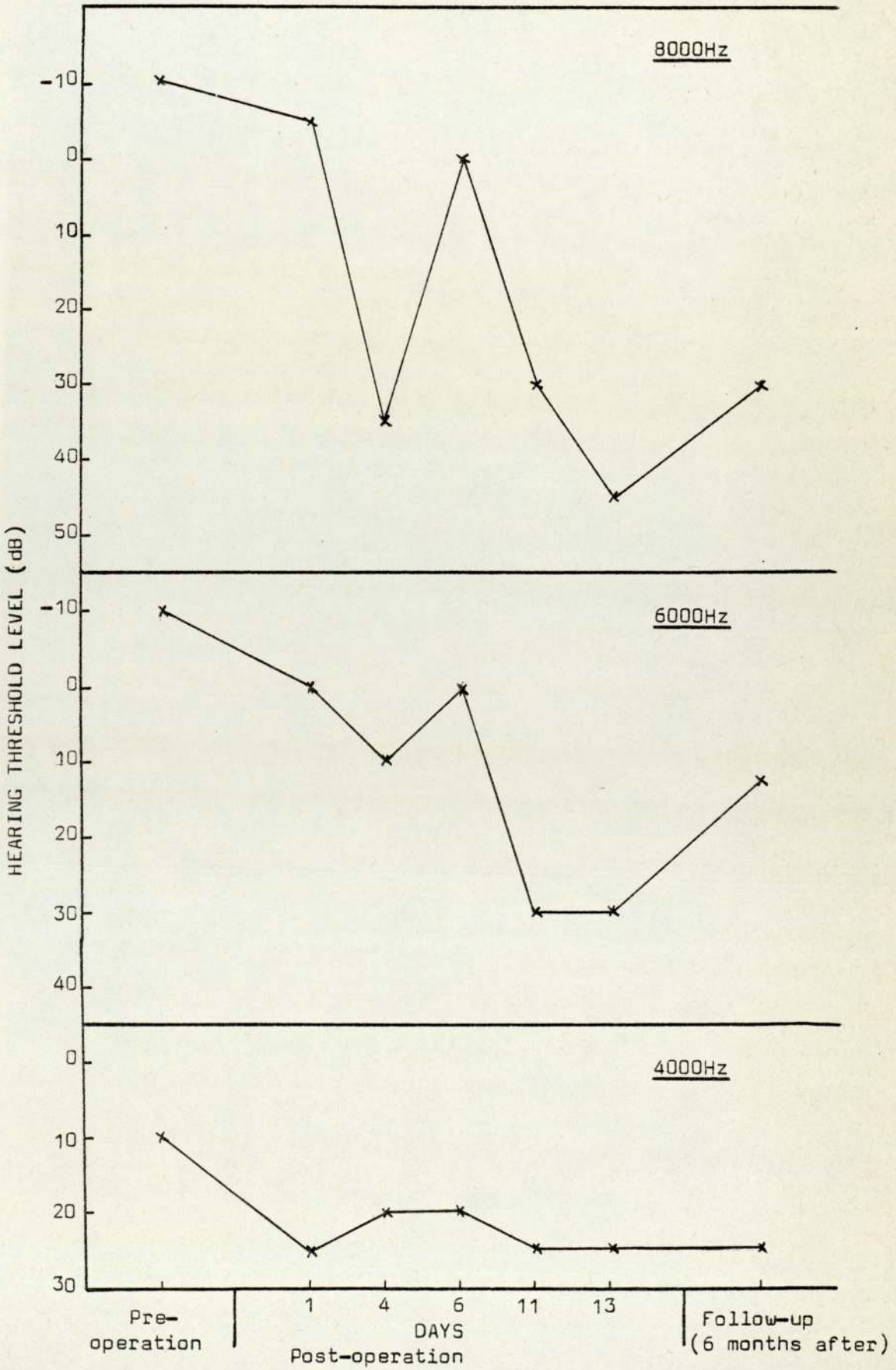
No firm conclusions can be drawn as to why these two patients had developed permanent unilateral hearing losses after surgery. Both of them received (β-lactam) antibiotics and in both cases nitrous oxide had been used as an anaesthetic during surgery. There was no drop in systolic blood pressure during or after the operation. Waun, Sweitzer and Hamilton (1967) observed hearing losses in patients following nitrous oxide anaesthesia. They hypothesized that the defect could result from 'changes in middle ear pressure incident to increased airway pressure and/or from differential solubility of nitrous oxide and nitrogen'. This change in differential solubility of the two gases may be prolonged and contributed to post-operative hearing losses if normal equilibrating mechanisms are not comprised.

Two patients in the control group showed an apparent improvement (gain) in hearing which was greater than 20dB. These patients were very ill when they were admitted to hospital and, therefore, when their baseline audiograms were recorded. As their condition improved, they were able to co-operate better with the test which was reflected in the audiogram results (Table 21).

4.3.2 Gentamicin-treated group

Of the 35 patients tested audiometrically, 22 (63%) had normal cochlear function before, during and after treatment. Baseline audiograms were obtained from 10 (29%) of 35 patients who received gentamicin. It was not possible

Fig. 17 Time course of the audiometric loss detected in the left ear of the control patient (case 2).



to obtain baseline audiograms from 25 (71%) patients because they were admitted to hospital during the night and started immediately on gentamicin.

6 (17%) patients died soon after or within six weeks of gentamicin therapy due to their underlying condition. Follow-up audiograms were obtained from 22 (76%) of the surviving patients. Follow-up audiograms were not obtained from 7 (24%) patients because six of them did not attend the clinic and one leukaemic patient was too ill to co-operate.

10 (24%) patients in this group demonstrated audiometric losses. Primary diagnoses in these 10 patients are listed in Table 22. Six patients in this group demonstrated fluctuating hearing losses or gains greater than 20dB which were similar to the type observed in three of the control patients. These hearing losses were observed at the time when the patients were very ill and the gains were observed as soon as they got better (Tables 21 and 22). Hearing losses in these patients (nos. 1 and 6) were therefore not considered to be drug-related (Table 22).

Audiometric losses detected in three patients (nos. 7, 9 and 10) were considered to be drug-related (Table 22). The condition of these three patients was quite good throughout the course of audiometric testing and therefore they gave full co-operation. In two of these patients the losses persisted and in one patient the loss was transient. Transient hearing loss occurred in patient no. 10 (Table 22). This loss is illustrated in Figure 18.

Symptomatic hearing decrease occurred in two patients (Table 22). In both cases (nos. 7 and 8), the losses were

Table 22 continued

Patients with symptomatic hearing losses

Patient No	Sex	Age	Severity of Illness	Primary Diagnosis	Reason for Gentamicin	Effect	Total Dose (g)	Duration of Therapy (days)	Previous Courses of Aminoglycosides
7	F	33	Fair	Acute Promyelocytic leukaemia	Febrile	Audiometric loss; Hearing decrease; Tinnitus	1.05	5	Three courses of gentamicin one month earlier; Previous total dose = 4.4 g
*8	F	60	Fair	Carcinoma of of bronchus	Febrile	Audiometric loss; Hearing decrease; Dizziness; Ataxia	1.60	7	None
Patients with drug-related non-symptomatic hearing losses									
9	M	49	Fair	Bronchiectasis	Pseudomonas	Audiometric loss	2.30	10	None
10	F	68	Fair	Perforated colonic diverticulum	Prophylaxis therapy	Audiometric loss; Ataxia	2.10	7	None

Audiometric losses recorded in patients nos. 7, 9 and 10 were considered to be drug-related.

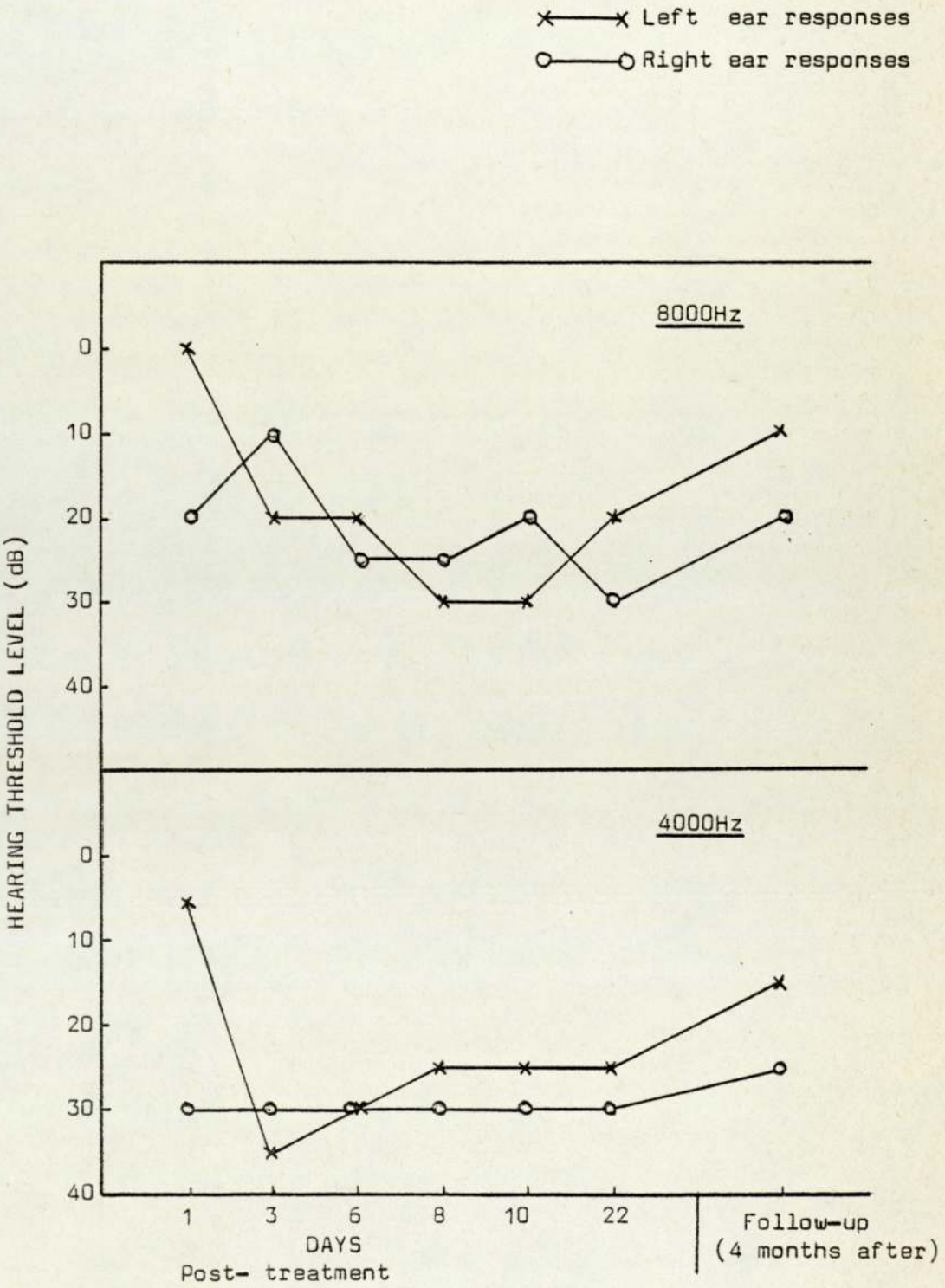
*Auditory effects observed in this patient were due to inappropriate ADH secretion.

Table 22 continued

Patients with symptomatic auditory effects without hearing losses

Patient No	Sex	Age	Severity of Illness	Primary Diagnosis	Reason for Gentamicin	Effect	Total Dose (g)	Duration of Therapy (days)	Previous Courses of Aminoglycosides
11	M	55	Fair	Urinary tract infection	Proteus mirabilis	Tinnitus	2.03	7	None
12	M	41	Critical	Aspiration pneumonia; Bacterial infection	Escherichia coli	Tinnitus; Dizziness	4.00	8	None

Fig. 18 Transient bilateral hearing loss demonstrated by patient no. 10.



also detected audiometrically but were considered to be drug-related in only one patient (no. 7). The temporary hearing loss in the second patient was considered to be due to inappropriate antidiuretic hormone (ADH). These patients are discussed fully under Section 4.3.3.

Patients nos. 11 and 12 did not show any hearing losses (Table 22) but they did demonstrate symptoms of inner ear dysfunction. In the case of patient no. 11, tinnitus was the only symptom recorded while in patient no. 12 tinnitus and dizziness were recorded. In both cases the symptoms appeared during therapy and they subsided soon after the gentamicin treatment had been stopped. However, the baseline and the follow-up audiograms performed six months later were normal in both the patients.

4.3.3 Symptomatic loss in gentamicin-treated patients

Symptomatic hearing decrease occurred in two patients (nos. 7 and 8) during therapy (Table 22).

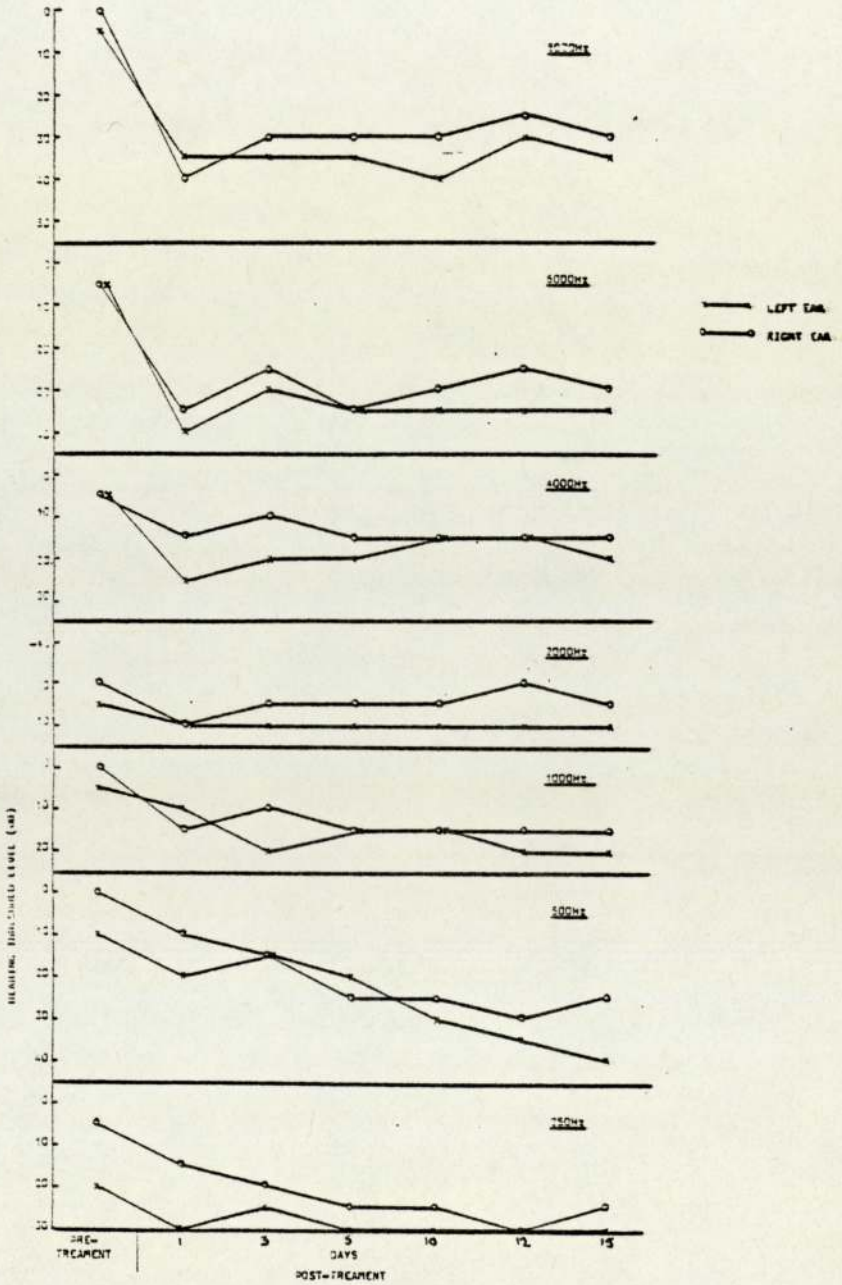
Patient no. 7, with a normal baseline audiogram, complained of tinnitus and was unable to hear speech properly after three days of gentamicin therapy. This was confirmed audiometrically by the presence of a moderate bilateral sensorineural hearing loss at all frequencies. Treatment with gentamicin was stopped on day 5 because of increasing subjective hearing loss and tinnitus. The hearing loss as detected by audiometry persisted at the last audiogram which was recorded four weeks after therapy had ended. The audiogram showed a bilateral mild hearing loss probably sensorineural in nature, at both low (250 and 500Hz) and at high frequencies (6000 and 8000Hz). The symptoms at

this stage were not present. The patient felt better, and was not aware of this mild hearing loss as it did not involve all the speech frequency range (500 - 2000Hz). The losses in this patient were considered to be drug-related as her hearing loss coincided with gentamicin therapy and at the time when these tests were conducted, she was well (Fig. 19). Also, she had received three previous courses of gentamicin with a one month interval between the last course and the present one.

Patient no. 8, a 60 year old woman, demonstrated a severe bilateral conductive hearing loss when a baseline audiogram was performed. She had satisfactorily been using a hearing aid for the past 20 years. Generally, at the time of gentamicin therapy she was ambulant but on the fourth day of therapy, she started to complain of unsteadiness and dizziness. On day 6 she was unable to hear even with the hearing aid turned on maximum and was very unsteady. This hearing loss was confirmed audiometrically. At the end of day 7, treatment with gentamicin was stopped due to increasing hearing loss and dizziness. Hearing loss persisted for 11 days after gentamicin therapy had been stopped and during this time the patient was unable to hear or walk without support.

The transient hearing loss observed in this patient was not considered to be drug-related but due to inappropriate ADH secretion. This resulted in the disturbance of her water and electrolyte balance. This phenomenon has been observed by Ransome and Ballantyne (1966). They reported that water intoxication caused an unusual increase in the volume of body fluid in their patients. They explained that

Fig. 19 Bilateral hearing loss demonstrated by patient no.7.



this disturbance of water and electrolyte balance could bring about osmotic damage to the sensory hair cells and so finally precipitate the observed hearing loss and ataxia.

In this patient (no. 8) the onset of her hearing decrease and the unsteadiness coincided with the inappropriate ADH secretion and once this had been resolved, the hearing level was restored to its original level. Therefore, it was assumed that the onset of the hearing loss and ataxia was associated in time with water intoxication which may have produced osmotic injury and so resulted in inner ear dysfunction. A follow-up audiogram was not performed on her as she died soon after discharge from the hospital.

4.3.4 Tinnitus

This occurred in three patients (nos. 7, 11 and 12) and subsided soon after treatment had been stopped (Tables 22). This effect was thought to be drug-related as none of the patients in the control group complained of this symptom.

4.3.5 Vestibular effects

Dizziness and ataxia subsided in all patients in whom they occurred (Table 22). All vestibular effects were considered to be related to gentamicin as none of these symptoms were observe in the control patient group. Only in patient no. 8 was it not considered to be drug-related as it was due to inappropriate ADH secretion.

Patient nos. 10 and 12 reported these symptoms. In patient no. 10 ataxia was observed during therapy but subsided soon after therapy. This patient also had a hearing loss which was considered to be drug-related.

Patient no. 12 complained of dizziness on the third

day of gentamicin therapy and this subsided within 17 days.

In this study, a very small group of gentamicin-treated patients were studied, therefore one cannot justify by drawing conclusions on the risk factors. Firm conclusions regarding risk factors can only be drawn when a very large group of patients have been studied. Hence, no real attempt was made to correlate the losses observed in the present study in three patients in the non-toxic group.

4.3.6 Onset of hearing loss

No case of sudden deafness occurred. Hearing loss developed gradually and could be followed by audiometry.

4.3.7 Serum levels of gentamicin

Measurement of gentamicin blood levels showed that six patients had peak levels greater than 10 µg/ml on one occasion. However, only one patient (no. 7) demonstrated ototoxicity. Trough levels were lower than 2 µg/ml in all patients except for three patients in whom they were observed to be greater than 2 µg/ml on one occasion. In the later case, the trough level was thought to be not true but was probably due to a missed dose which was given 4 h later. The level at 4 h after the next dose was determined to be the same as the trough level which was determined before the dose was given. However, none of these patients demonstrated any toxicity.

4.3.8 Renal impairment

Data on values of BUN, serum creatinine, creatinine clearances and urinary electrolytes were evaluated for determination of renal function which was found to be normal in all the ototoxic patients before and after therapy. Four patients showed an elevation of serum creatinine during

treatment with elevated serum levels but they did not demonstrate any ototoxicity.

In the gentamicin-treated group, there were only two patients who had renal impairment before gentamicin therapy. Their renal dysfunction was known at the time of gentamicin administration and the doses were adjusted accordingly.

4.4 Discussion and Conclusions

Three patients among the 35 treated with gentamicin were found to have incurred ototoxicity. Transient audiometric losses, which reversed after two days, in patients numbered 1 to 6 (Table 22) were considered to be of doubtful relationship to gentamicin since such losses were also detected in the control patient group. These losses coincided with the seriousness of the condition of the patients at the time the tests were performed and reversed as soon as they were feeling better. When the six questionable audiometric losses were excluded then the patients demonstrating ototoxic effects could be classified as follows:

Table 23: Classification of the hearing losses in the gentamicin-treated patients

	Patients
Audiometric losses only	1
Audiometric loss + hearing decrease + tinnitus	1
Audiometric loss (transient) + ataxia	1
Tinnitus only	1
Tinnitus + dizziness	1

In two patients, the drug-related auditory losses persisted but in one patient the loss was only transient. One patient had tinnitus and another patient demonstrated both tinnitus and dizziness. Tinnitus and dizziness subsided as soon as treatment with gentamicin had been stopped.

The first changes observed in the audiograms (Black et al., 1976) were losses at high frequencies (6000 and 8000Hz). the low frequencies (250 and 500Hz) were affected after this

(Fig. 19). Patterns of hair cell destruction from animal studies (Wersäll and Lundquist, 1968; Kohonen, 1965) indicate that outer hair cells of the basal turn are the sensory elements most sensitive to aminoglycosides. Damage then proceeds apical ward in the case of the outer hair cells. Inner hair cells are affected initially at the apex and later at the base. The characteristic behaviourial effect, therefore, has been reported to be rise in threshold for the high frequency pure tones which are transduced into nervous signals by the sensory elements of the basal portion of the organ of Corti. Thus, there is a good agreement between morphological damage to the basal outer hair cells and the functional disruption of the frequencies they serve.

Hawkins (1959) provided further evidence that the hair cells suffered early damage when he demonstrated that the hair cell potential, the cochlear microphonic (CM), diminished in amplitude soon after treatment with kanamycin, streptomycin and viomycin while the N_1 action potential remained intact.

Since the audiometric tests in the present study were conducted on the ward where there was considerable background noise, a 20dB loss at any frequency which was sustained was used in order to qualify as auditory toxicity. This was chosen to compensate for any variability in the audiograms which may be encountered when such tests are applied on the ward and not in the sound-proofed booth. Follow-up audiograms were performed in the sound-proof booth in the ENT Department. Control studies using 24 normal subjects of both sexes (Section 3.4, Page 70) showed that significant differences between the ward and the sound-proofed booth occurred at

the low frequencies namely 250 and 500Hz while the higher frequencies were unaffected. Aminoglycosides have been reported to affect higher frequencies initially, therefore, the low frequencies are not so important when one is interested in detecting the earliest otological changes before the patient is affected symptomatically.

Introduction of new aminoglycosides provoked much interest in the relative toxicities of these drugs. It is unfortunate that lack of commonly accepted criteria for defining ototoxicity makes valid comparisons difficult in human studies. Most of the reported studies (Black et al., 1976; Smith et al., 1977; Fee, Vierra and Lathrop, 1978; Bender et al., 1979) have compared baseline audiograms with the end of treatment audiograms. The present study has shown that it may not be valid to compare the first audiogram with the end of treatment audiogram. Very ill patients cannot cope well with the audiometric tests. Losses detected in such patients should be interpreted with caution. Other patients have shown an apparent improvement in hearing at the end of treatment. Four such patients were observed in the gentamicin-treated group and three in the control patient group. None of the above studies had considered the effect of illness on the performance of audiometry and had not incorporated any control patients. Very narrow limits were adopted by some workers who considered a hearing loss as small as 10dB at any frequency to represent changes related to therapy (Smith et al., 1977; Panwalker et al., 1978).

The studies conducted using normal subjects (Section 3.5, Page 81) indicated that there were no differences between

two audiograms performed consecutively on two days on the ward using the portable audiometer. Thus in the ototoxicity studies, any auditory changes detected by comparing the baseline or initial audiogram with subsequent audiograms performed during or after treatment, either reflected a real change in hearing or the inability of the patient to co-operate during one or more tests. The patient control group showed that although alertness and co-operation of the patient is essential, in most cases there was a good correspondence between the serial audiograms of the same patient. Three patients in the control group (Table 21) demonstrated audiometric changes when their general condition was not good. These changes reversed within two days when the patients were feeling better. In the gentamicin-treated group, six patients showed similar changes and were thus not considered to be drug-related.

The two patients, with persistent drug-related auditory losses, did not differ from the remainder of the patients in the same group. One of these two patients had received three courses of gentamicin previously and also had a high serum peak level (14.8 µg/ml) on one occasion. However, other patients with many risk factors, did not develop any ototoxicity. One patient who did not demonstrate any ototoxicity had received four courses of gentamicin, two of streptomycin and one each of viomycin and kanamycin. The absence of risk factors, on the other hand, did not preclude the occurrence of permanent unilateral hearing loss in one patient (Table 22).

Existing hearing loss evidently did not contribute to a further loss. Of the 15 patients with a degree of existing hearing loss, only one patient developed a further hearing loss

during therapy. Two of the 20 patients with normal hearing developed a hearing loss.

The total dose of gentamicin and the duration of treatment did not correlate with the incidence or severity of hearing loss because the doses and the durations were almost equal in both the toxic and the non-toxic groups.

Furthermore, it was determined whether any patient had a history of exposure to excessive noise over a protracted period or whether hearing impairment had been present in parents or grandparents. Previous exposure to noise and the probability of inherited proneness of hearing loss were not present in the ototoxic patients.

Jackson and Arcieri (1971) reported that advanced age did not appear to contribute to ototoxicity in the patients studied by them. The present data showed the same negative finding. Earlier, Jackson (1967) had considered advanced age to be an important contributory factor to gentamicin ototoxicity. In Jackson's study the 70 patients suspected of incurring ototoxicity had a significantly older mean age than the 843 controls. This was accounted for by the relative infrequency of ototoxicity demonstrated in children. However, when a matched-test sample of 219 patients was used, no significant difference in age was found between those with ototoxicity and controls.

In the present study one or more vestibular effects (dizziness and ataxia) occurred in three patients during therapy and subsided when therapy ended.

In three patients tinnitus occurred. In one case, when tinnitus was present together with hearing loss, both appeared

on the same day so tinnitus could not be considered as a warning of impending hearing decrease or audiometric loss.

Damage to the sensory cells of the inner ear should be regarded as a permanent effect because the hair cells are non-regenerative, although there may be some improvement in function. Once the audiometric evaluation shows a depression in hearing, it may be too late for any effective action. Immediate withdrawal of the drug and even removing the drug from the blood by dialysis may still leave sufficient drug in the inner ear fluids to cause progressive or even total destruction of the inner ear.

The route by which aminoglycosides reach the sensory hair cells of the organ of Corti is presumed to be via the modiolar vessels going to the capillary loops beneath the basilar membrane but it has not been well established and conflicting results have been reported to date (Konishi, 1979).

Stupp, Küpper, Lagler, Sous and Quante (1973) investigated the pharmacokinetics of aminoglycoside antibiotics administered systemically. Their qualitative bioassays indicated that high concentrations of the antibiotics were present in the perilymph with concentrations in the endolymph being almost as high. The half-life of neomycin was about 15 h in the inner ear fluids, being 10 times longer than the half-life in the blood. They concluded that the organ specificity of the antibiotics was as a result of accumulation in the inner ear fluids with a slow efflux out of the perilymphatic space.

Balogh, Hiraide and Ishii (1970) studied the distribution of tritiated dihydrostreptomycin injected intraperitoneally in guinea-pigs. They reported that tritiated dihydrostreptomycin

reached higher concentrations in the perilymph than in the endolymph. From their observations they assumed that dihydrostreptomycin probably entered the perilymphatic space from the spiral ligament and that the antibiotic reached the endolymph either directly from the stria vascularis or from the perilymphatic space.

Illberg, Spoendlin and Arnold (1971) perfused the perilymphatic space with tritiated dihydrostreptomycin solution. Their results indicated that the specific sensitivity of the hair cells to dihydrostreptomycin was not only due to the long persistence of the substance in the perilymph but also due to the specific affinity of the drug to their cell membranes.

From autoradiographic studies and bioassays of the distribution of aminoglycosides the gradual suppression of the CM and the N_1 action potential was observed during the perfusion of the perilymphatic space with these antibiotics. This suppression of potentials can be explained if the drugs reach the endolymph as suggested by Balogh et al. (1970). A delay in the suppression of the CM by intraperitoneal administration may reflect the time which is necessary for the drugs to reach the critical level before the CM is substantially suppressed.

There are various contradictory reports of the incidence of gentamicin ototoxicity. In a retrospective study, Jackson and Arcieri (1971) examined case histories of 1,484 patients treated with gentamicin during 1966 to 1969. The incidence of gentamicin-induced ototoxicity for the four year period was reported to be 2%. In two-thirds of the patients the

dysfunction was limited to the vestibular system; one-third had a change in hearing ability. Among the latter, vestibular symptoms were also present in one-half of the group. Impaired renal function was present in a large proportion of those patients who showed toxicity. However, Nordström et al. (1973) in their prospective investigation on the ototoxicity of gentamicin reported an incidence of vestibular toxicity of 10%. The discrepancy between the two studies was explained by Nordström et al. (1973) to be partly due to the different ways the incidence was expressed together with differences in design and material in each of the investigations. In Nordstrom et al.'s study there was a high percentage of patients with impaired renal function. Furthermore, the age of the patients was relatively high and 50% of those who demonstrated pathological changes had received two courses of gentamicin. All these factors could have contributed to the differences in frequency of ototoxicity between the two studies although the effect of age was shown not to be statistically significant (Jackson and Arcieri, 1971).

In the present study, three of the 35 gentamicin-treated patients demonstrated audiological abnormalities giving an incidence of 8.6%. The present study does not justify any conclusions to be drawn concerning risk factors since the patients were a very small group in whom therapy was closely monitored and adjusted. In order to draw firm conclusions, a study should comprise of a very large group of patients being treated with gentamicin and also a group of carefully matched controls should be included. None of the other studies mentioned above had looked at control patients who were

not on aminoglycoside therapy. Their studies were based on comparing the results from the toxic group with those of the non-toxic group. It is of utmost importance to evaluate the changes of audiometric tests in ill patients before analysing the results of aminoglycoside treated patients.

Finally, individual susceptibility to gentamicin seems to vary widely, for which reason it is difficult to assess the risk of side-effects in any given case. In this study despite careful dosage and close observation, ototoxic effects were not avoided.

SECTION 5

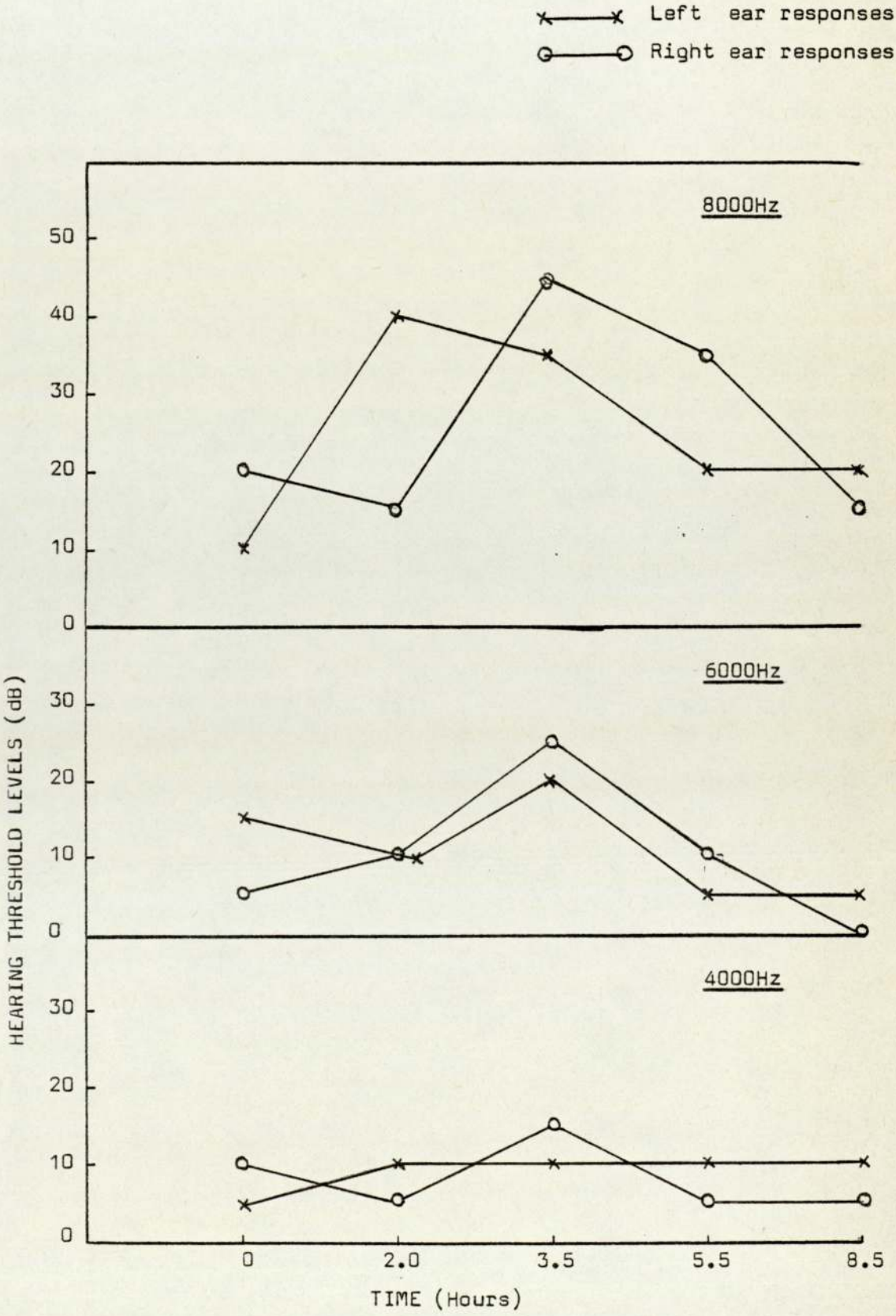
5.1 PRELIMINARY STUDIES ON THE ACUTE OTOTOXICITY OF GENTAMICIN

Two normal healthy male volunteers, who were being studied for other reasons, were administered an intravenous dose of 120 mg of gentamicin which was infused over a period of 5 min. Pure-tone air conduction tests were performed before gentamicin administration and then every 2 to 2½ h post dose using the Kamplex portable audiometer in the same manner as mentioned in Section 3 (Page 45). Blood samples were taken for the determination of serum concentrations every half hour for the duration of testing.

In both these subjects a transient mild bilateral high frequency loss was observed. One of these subjects gave inconsistent responses when tested again the next day. Therefore, the increase in threshold observed in this subject was not considered to be of significance. In the second subject the increase in the hearing threshold was observed after 3½ h of infusion but this loss reversed back to the original level after 5½ h (Fig. 20). Despite the changes observed audiometrically at high frequencies (6000 and 8000Hz), the subject did not complain of any hearing loss. This may be due to the fact that the loss was not affecting the speech frequency range and was also mild.

Other workers have employed audiometry in detecting hearing losses (Dobbs and Mawer, 1976; Wilson and Ramsden, 1977). They reported no hearing loss. One of the reasons for this could be that they were not looking for acute changes due to gentamicin with audiometry and so were not testing at the time intervals employed in the present study.

Fig. 20 Changes in hearing thresholds observed in one subject after an intravenous injection of 80 mg of gentamicin.



Dobbs and Mawer (1976) studied the effect of intravenous injections of 80 mg of gentamicin or tobramycin given within 1 min to six healthy volunteers on the hearing thresholds. Pure-tone audiometry was performed before the administration of the drugs and then post-dose at the time intervals of 1 h, one week and one month. The maximum concentration of the antibiotics in serum, which were noted 2 to 5 min after injection, ranged from 9.3 to 15.5 μg/ml for gentamicin and from 7.4 to 12.9 μg/ml for tobramycin. Despite these high concentrations, they were unable to detect loss of hearing at any frequency at any time after the injection of antibiotics.

Wilson and Ramsden (1977) did not observe any auditory symptoms after tobramycin administration in the three patients studied although immediate reduction in cochlear output was observed by electrocochleography. Audiometry was performed by them before, during and after treatment. However, the times when the tests were conducted post-dose were not stated.

The fact that a transient hearing loss was observed in one of the normal subject's posed two important points. These were:

1. Did this increase in threshold occur in every person after a short interval of gentamicin administration.
2. If this acute effect occurred in every person then it was probably of no importance, but if it occurred in only a few people then these people may be the ones that are at risk from gentamicin therapy. If the latter is the case, then it would be helpful to pick out these patients who are on gentamicin therapy by performing audiometry at hourly intervals after the first dose of the drug. These patients

could then be kept under close observation and if any hearing or vestibular symptoms are demonstrated then the drug be stopped.

To answer these questions, some more individuals had to be tested. Volunteer subjects could not be used since it would be unethical to expose the normal subjects to gentamicin if this acute effect was real and it occurred in every person. Therefore, leukaemic patients who were on gentamicin therapy were used. These patients were well and were able to cope with audiometry as required.

Seven patients (6 male, 1 female) were studied. Pure-tone air and bone conduction tests were performed before drug administration and then every hour for 6 h post-dose. Blood samples were taken for the determination of serum concentrations every half hour for the duration of testing. In four of these patients, the tests were carried out during the first dose while in the other three patients, the tests were performed after the second dose. None of these patients demonstrated any audiometric changes.

5.2 Discussion and Conclusions

A mild bilateral high frequency loss was observed after $3\frac{1}{2}$ h of gentamicin infusion in one subject. This was reversed after $5\frac{1}{2}$ h post-dose. However, none of the patients tested showed any changes indicating that this effect does not occur in every person. This fact could explain the wide individual variations encountered with these antibiotics. It may be that those people that show early changes in hearing threshold after a single dose are the ones that are more susceptible to aminoglycoside ototoxicity. If this is the case, then all patients on gentamicin or other aminoglycosides can be tested audiometrically every hour after the first dose of the drug. If any of the patients show these auditory changes, then these are the patients who should have their therapy monitored closely and they should have regular audiometric tests as well.

Although, the fact that aminoglycoside antibiotics have an inherent potential for causing ototoxicity has long been recognized, the mechanism of ototoxicity is unknown. Prolonged exposure of the inner ear to sharply elevated concentrations apparently results in damage to the neuroepithelial cells of the vastibular apparatus, semicircular canals and cochlea (Stupp et al., 1973). The peculiar toxicity of the aminoglycosides for only two organs of the body, namely, the inner ear and the kidney, has been proved difficult to explain. Aminoglycosides have an ability to affect many biological systems. For instance, they have been shown to inhibit protein synthesis. This is accomplished through an interaction with the 30S ribosomal subunit (Benveniste and

Davies, 1973). Recent work by Schacht (1976) has revealed an interference with the lipids of the cell membrane rather than with the synthesis of protein. Specifically, neomycin has been found to inhibit phosphoinositide metabolism, which is believed to be essential in the control of membrane structure and permeability. Incorporation of (^{32}P)-orthophosphate into the phosphoinositides of the organ of Corti and stria vascularis was decreased by daily parental administration of neomycin. In homogenates of these tissues the presence of neomycin blocked the hydrolysis of phosphatidylinositol diphosphate and inhibited the binding of calcium. Apparently, it was thought that neomycin occupied the binding site for calcium with part of its molecule. Schacht (1976) explained that this could disturb the membrane structure and the dephosphorylation/phosphorylation cycle can be interrupted and as a result, the cell is unable to perform its normal duties and sooner or later dies.

Hawkins (1970) suggested that the inhibition of protein synthesis by the aminoglycosides may cause destruction of the spiral ligament and Reissner's membrane whereas an effect on membrane permeability may cause immediate effects on hair cell potentials.

Much of the literature contains articles on the long term effects of aminoglycosides but the immediate effects of these drugs have been reported by only two workers (Logan, Prazma, Thomas and Fischer, 1974; Wilson and Ramsden, 1977). Both these studies were based on the primary effects of aminoglycosides on the cochlear function.

Logan et al. (1974) studied the effect of tobramycin on

the cochlear potentials of the guinea-pigs using electronystagmography. Three different doses (50, 100 and 200 mg/kg) of the drug were used and the results were compared statistically with the normal control animals. Tobramycin was infused into the contralateral jugular vein over a five minute interval to achieve the highest concentrations of the drug in the cochlea within a few minutes. For statistical evaluation, cochlear microphonics (CM) and whole nerve action potentials (AP) were calculated as percentage pre-infusion values.

They found that the changes produced by 50 mg/kg dose in CM and AP were not significant. The 100 mg/kg dose produced significant drops of CM and AP immediately after the infusion, with a continued decrease until the end of the experiment. However, the endocochlear potentials (EP) did not change significantly. The largest changes were observed with 200 mg/kg dose where the CM and AP dropped to zero and the EP became negative immediately after drug infusion.

This rapid action produced at the higher dose was explained to be probably due to an interruption of the cell respiration or blocking of the active-ion transport or both in the stria vascularis. At the lower doses, drug action on the stria vascularis may start later possibly after several doses or may have only a small effect.

Brummet and Brown (1975) critised Logan et al.'s (1974) work and indicated that the ototoxic lesion induced by tobramycin does not occur immediately after an intravenous dose as reported by Logan et al. Brummet and Brown (1975) showed that the changes in cochlear potentials occurred when the blood pressure was severely depressed and the potentials returned to pre-drug

administration level as soon as the blood pressure returned to normal. They reported that the immediate effects observed by Logan et al. (1974) were possibly due to a depression of blood pressure in their animals.

Immediate and reversible depression of cochlear activity in humans without clinical evidence of impairment has been shown to follow single intravenous doses of tobramycin (Wilson and Ramsden, 1977). Electrocochleography was performed on three patients to monitor intravenous administration of tobramycin. Immediate reduction in cochlear output was observed when peak serum levels exceeded 8 to 10 μg/ml. This cochlear output reversed to pre-administration levels as soon as the serum levels fell. They postulated that the speed of onset of these tobramycin effects suggested that a metabolic block was responsible at one or more sites in the cochlea. One possible mode of action is the interference with cation transport across membranes.

The reversal of the hearing loss observed in the present study suggests that the alterations in the cochlear function were temporary after a single injection of gentamicin, but with continued administration it may have become permanent. The reversal of hearing loss can be explained by the effect of gentamicin on the sodium-potassium pump. The composition of the endolymph is similar to that of the intracellular fluid, in that both fluids contain more potassium ions than sodium ions. This ionic balance presupposes the existence of an active-transport mechanism, such as the sodium-potassium pump, to maintain ionic differences between the endolymph and the perilymph. It is possible that a primary effect of the

aminoglycosides is on the interruption of the active-transport system, leading to alterations in the normal ionic concentrations of the labyrinthine fluids. These alterations may impair electrical activity to the point where the nerve conduction is interrupted. Thus, destruction of the hair cells and other neural elements may stem from changes in the osmotic gradient, brought about by the direct action of the aminoglycosides. Initial alterations of the mechanism that controls the gradient may be reversible; this finding would explain the reversible hearing loss observed in this study. However, there appears to be a point at which damage to the hair cells is so severe that it is irreparable.

SECTION 6

EVALUATION OF SOME OF THE METHODS USED TO DETERMINE PROTEIN BINDING OF GENTAMICIN

6.1 Introduction

The binding of drugs to serum proteins has attracted much attention since the earliest days of therapeutics. The interaction between a drug and protein molecules often profoundly influences its biological activity, for not only is such an association necessary for 'drug-receptor site' interactions and metabolism to take place, but it may also play a primary role in governing the drug's absorption, distribution and excretion characteristics. Serum protein binding appears to be one of the important determinants of distribution of drugs in the body (Davis, 1943; Anton, 1961; Kunin, 1962). A complete understanding of the nature and magnitude of drug-protein binding is thus fundamental to an accurate prediction of the therapeutic and toxic effect of drugs. Normally the drug will not express its biological activity when bound to serum proteins. The interaction of a drug with the serum proteins may limit its availability to the receptor sites and to the drug metabolism and excretory systems. These interactions are completely reversible and non-specific in a majority of cases. Hence, drugs that are weakly bound to proteins will be freely diffusible, whereas strongly bound drugs may influence the drug's potential bioavailability. Highly bound drugs tend to remain in the vascular compartment, giving high blood levels, while weakly bound drugs may diffuse more rapidly into the interstitial fluid. Binding to serum proteins may delay uptake and metabolism of drugs by the liver and slow renal excretion (Brauer and

Pessotti, 1959; Anton, 1961).

Drug binding to proteins or to macromolecules in body fluids can reduce its effective concentration, decrease its antibacterial activity and modify its pharmacokinetics or toxicity. Data in the literature regarding the extent of gentamicin binding to serum proteins is conflicting with reports of percentage drug bound ranging from 0 to 70% (Bulger, Sidell and Kirby, 1963; Riff and Jackson, 1971; Gordon, Regamy and Kirby, 1972; Ramirez-Ronda, Holmes and Sanford, 1975; Myers, Bennett and Olsen, 1976; Myers, DeFehr, Bennett, Porter and Olsen, 1978). Part of the variation between studies can be accounted for by the concentrations of calcium and magnesium in the test system (Ramirez-Ronda et al., 1975; Myers et al., 1978). Ramirez-Ronda and co-workers (1975) reported zero protein binding of gentamicin under physiological conditions and, further, showed an inverse relationship between bound drug and the concentration of ionized calcium and magnesium. They suggested that failure to control the divalent cation concentration precisely might be responsible for the conflicting reports. The results of the studies conducted by Myers et al. (1978) agreed with those of Ramirez-Ronda and associates (1975) in that they demonstrated enhanced gentamicin binding in the absence of divalent cations, but 20% of binding was observed by Myers et al. under physiological conditions. The reasons for the varying results were unclear since experimental design was similar although Myers et al. used ^{14}C rather than ^{3}H to label gentamicin.

Hoffman and Wood (1977) conducted a prospective study on sixty patients receiving gentamicin to determine the incidence of nephrotoxicity and to study the influence of several variables

on the potential for developing gentamicin associated nephrotoxicity. The variables studied were patient's age; total grams of gentamicin received; total number of days the patient received gentamicin with a haemoglobulin of less than 12 g%; sex; total days duration of therapy; haemoglobulin prior to therapy; haematocrit prior to therapy; red blood cell count prior to therapy; albumin level prior to therapy; and concomitant therapy with gentamicin and other potentially nephrotoxic drugs. Seventeen patients were excluded from the study due to missing variables. Of the remaining patients, ten were classified as 'toxic' and twenty-three were classified as 'non-toxic'. The incidence of nephrotoxicity was determined to be 16.7%. Data relating to the variables identified were analysed by Hoffman and Wood (1977) by the utilization of chi-square , t-test and multiple regression analyses. Two variables were found by them to be highly significant in relation to the development of gentamicin-induced nephrotoxicity. These were the albumin level prior to therapy (lower albumin levels in the 'toxic' group) and the concomitant use of another potentially nephrotoxic drug. They concluded that the mechanism behind the influence of albumin on gentamicin toxicity was unclear, but was probably related to protein binding.

The theory behind the relationship between low albumin levels and nephrotoxicity secondary to gentamicin can be explained by significant gentamicin binding to the serum proteins. With less binding sites due to low levels of albumin, more free gentamicin would be available leading to a greater potential for toxicity. The binding of gentamicin to plasma proteins would limit its concentration in tissues and its locus of action,

since only the unbound gentamicin would be in equilibrium across the membranes. Binding will also decrease glomerular filtration of gentamicin, but will not affect renal tubular secretion (Hoffman and Wood, 1977).

Riff and Jackson (1971) found that there was an inverse relationship between the peak concentration of gentamicin and the patient's haematocrit. They postulated that if red blood cells could absorb and release gentamicin in some equilibrium with plasma, then this would lower the peak concentration. It would also protect the drug from rapid excretion and prolong the duration of low levels after the dose. They recommended dosage alteration depending upon the patient's haematocrit to avoid ototoxicity.

The protein most generally involved in drug interaction is albumin, but the belief that globulins play only a minor role may require revision, particularly at low concentrations of drug. Thus α-globulins with very high affinity for cortisol and thyroxine have relatively little total binding capacity for these hormones. Only at a relatively high concentration, when the globulin binding capacity is saturated, they are taken up by albumin. The binding at such sites with high affinity can be easily overlooked if the method of estimation of the drug is insensitive. Electrophoretic separation of serum containing tracer amounts of radioactively labelled compound represents one method of identifying binding to minor protein fractions.

Apart from neutral, lipid-soluble drugs that can be associated with the globulins of lipoprotein complexes by mere solution in the lipid component, binding consists in the interaction of ionized, polar, or non-polar groups of a drug

with corresponding groups of the protein. The energy of the binding depends upon the number and nature of these interactions involved at each binding site. The studies by Klotz (1957) have indicated the complementary relationship between amino acid composition of a protein and its ability to bind molecules of a particular structure or charge. Thus the relatively high affinity of albumin for anions, inspite of its net negative charge at pH 7.4, is explicable in terms of the hydrogen bonds formed preferentially between the hydroxyl and carboxy groups of amino acids, serving to leave unbonded cationic nitrogen groups (of lysine particularly) available for binding with anions. The abundance of hydroxy amino acids in albumin, relative to its carboxy amino acid content, sets it apart from other proteins, and contributes to the availability of cationic groups for binding; although the folding of the molecule is another contributory factor. Even so, the number of primary binding sites of albumin for organic anions is generally no more than five, the number of secondary sites being about twenty.

Little information is available on potential binding sites for basic compounds in either bovine serum albumin or human serum albumin. It is generally thought that albumin has different binding sites for acidic and basic drugs (Brodie, 1965). Basic drugs never replace acidic drugs at the same site.

6.1.1 Significance of plasma protein binding

6.1.1.1 Absorption

A high degree of plasma protein binding could enhance the intestinal absorption of a drug by rendering its concentration gradient favourable for absorption. This may be of particular importance with drugs having a poor water solubility, since it

will enable larger concentrations of drug to be carried in the plasma (Brodie, 1965). Unfortunately there are few reports to substantiate the role of plasma protein binding in influencing intestinal absorption.

6.1.1.2 Distribution and pharmacological activity

Most drugs probably distribute through the body water and tissues by passive distribution down a concentration gradient. Generally, it is considered that only the unbound drug in the plasma is available for transport to extravascular sites (Goldstein, 1949; Brodie, 1965). Among the experimental observations supporting this view are the reduced potency of drugs such as sulphonamides (Anton, 1960) and penicillins (Rolinson and Sutherland, 1965) when bound to serum albumin.

It is by no means certain that a protein bound drug is invariably therapeutically and toxicologically inactive. Providing they do not bind preferentially or irreversibly to tissue sites, highly bound drugs are likely to be located initially in the plasma compartment. Under these circumstances the protein bound drugs can serve potentially as a reservoir replenishing by dissociation some of the drug that is lost by metabolism and excretion, thus tending to maintain the concentration of unbound drug at a therapeutically useful concentration. Plasma protein binding could also reduce the free concentration of some drugs below that required to elicit a toxic response, thus rendering the drug safe for therapeutic use. Plasma proteins may thus have an important buffering role for many drugs, enabling them to be given only a few times a day instead of by continuous infusion.

Although tissue binding has not yet been investigated

extensively, it seems likely that some highly bound drugs have even high affinities for certain tissue proteins. An example of this is the cardiac glycosides. It has been found *in vitro* to have a higher affinity for actin and myosin than for bovine-serum albumin (Genazzani and Santamaria, 1969), such that *in vivo* their concentration in the heart is about twenty or thirty times that in plasma. From the above considerations it is apparent that it is usually only the free drug and the drug metabolite concentration in the plasma which could be expected to directly correlate with the therapeutic or toxicological effects of the drug.

6.1.1.3 Excretion

The removable of gentamicin and other aminoglycosides is principally through renal excretion by glomerular filtration. Recovery of gentamicin is nearly complete in patients with normal excretion (Jackson, 1977). This suggests that metabolism in man is negligible when renal excretion is rapid. However, when renal excretion is reduced, gentamicin may be distributed in a larger space, probably due to slow penetration into the intracellular compartment (Gyselynck, Forrey and Cutler, 1971). These workers reported that the renal clearance of gentamicin did not differ significantly from inulin but was lower than the clearance of creatinine. They found that 25% of gentamicin was bound to serum proteins. It was suggested by them that the 75% of the 'free' gentamicin in serum was ultrafilterable. Their data on clearances indicated that there was a nett tubular secretion of gentamicin in addition to glomerular filtration.

6.1.1.4 Competition of binding sites

Binding of an exogenous compound to plasma proteins may

potentially increase or decrease the protein binding capacity for other endogenous and exogenous compounds. Alternatively an increase in levels of endogenous compounds may lead to changes in drug binding. Displacement phenomena are most frequently noted. Odell (1959) demonstrated that bilirubin can be displaced from serum albumin by sulphonamides with resultant toxicity. This displacement is of particular significance in premature babies with low plasma-albumin concentrations or in individuals with an impaired capacity to metabolize bilirubin. Numerous drugs have been shown in vitro and in animal experiments to compete for plasma protein binding sites (Meyer and Guttman, 1968) and many potential drug-drug interactions have been reviewed (Hussar, 1969; Sher, 1971). Competition between compounds for protein-binding sites is likely to be of particular importance when the drug's distribution volume is small. Competition between compounds for protein-binding sites is likely to be of particular importance when the drug's distribution volume is small. If the drug on displacement is readily taken up by other tissue-binding sites then its biological efficacy may not be significantly modified. The data relating to human subjects is unfortunately limited. This type of interaction has been suggested to be the cause of clinically undesirable side effects in a number of instances. The most frequently cited cases are the displacement of the anticoagulant drug warfarin by highly bound drugs such as phenylbutazone, indomethicin, salicylate, fatty acids and various sulphonamides (Solomon and Schrogie, 1967) from both plasma and tissue-binding sites, such as the liver, leading to increased anticoagulant effect which may give rise to spontaneous haemorrhage. Drug

displacement might in certain circumstances be of potential clinical value. Thus highly albumin-bound pharmacologically relatively inert excipients such as sodium trichloroacetate, which lower the dose of a drug necessary to produce a given pharmacological response, might be included in a pharmaceutical preparation of a drug such as warfarin in order to give stable blood levels even when other highly bound drugs known to displace warfarin are concurrently administered. Because of the complexity of protein binding and our lack of a thorough understanding of it, this approach could only be used in special controlled circumstances. It is unfortunately difficult to predict the displacement of one drug by another merely from a knowledge of binding data and plasma concentrations.

A drug with a higher association constant does not necessarily displace a drug of lower affinity unless both share common binding sites.

6.1.1.5 Binding of drugs to tissues

The major contributor of competitive protein binding with blood constituents is undoubtedly tissue protein binding, unfortunately knowledge of this binding is still in a primitive state compared with that of albumin binding. Binding of drugs to plasma proteins has been extensively studied primarily because plasma is readily accessible to sampling, can easily be separated into its constituent macromolecules, and drug-protein interactions are easily quantitated. Tissue binding studies have none of these advantages and as a result, knowledge of the qualitative and quantitative aspects of the binding of drugs to tissue components is poorly understood. Since the mass of tissue is greater than the albumin mass, it is apparent that for

many compounds tissue binding may be of more impotance than albumin binding. It is usually assumed that the fraction of unbound drug in the tissues is the same as the unbound fraction in the plasma. However, the situation *in vivo* may be complicated by binding to tissue proteins, which will entail a series of equilibria being established between the tissues of various organs and the tissue fluids. Tissue uptake after a single dose of a drug may be rapid, disappearance is often slow (Wagner, 1973). Kunin (1965) demonstrated that the distributions of penicillins in the tissues of the rabbit was inversely related to their known binding to rabbit serum.

Binding may occur to the cell-membrane nucleus, other cytoplasmic organelles or cytosol. This binding may be with proteins, polypeptides, lipids or polysaccharides. It is likely that a number of specific and non-specific cellular binding proteins will be isolated and characterized in the future which will enable a more sophisticated interpretation of tissue-binding phenomena.

6.1.2 Factors which alter drug-protein binding

6.1.2.1 Protein concentration

Many diseases and physiological alterations of the body cause changes in protein concentrations of the plasma and extravascular compartments. The inter-relationship between per cent binding, protein concentration, and drug concentration is non-linear and complex. Drugs which are weakly bound ($k = 10^4$) show changes in binding which are somewhat proportional to the protein concentration, particularly over a physiological range of protein concentrations ($10^{-5} - 10^{-3}$M). Drugs with a strong association constant ($k = 10^7$) are at the other extreme.

Appreciable changes in protein concentration do not alter the degree of drug binding over an extensive range. However, at low concentrations of protein (less than 0.2×10^{-4}M), there is a substantial difference in binding which is dependent on the drug concentration. Drugs with an intermediate association constant ($k = 10^5$) show appreciable dependence on both the drug and protein concentration in the degree of binding.

Many pathophysiological states cause a change (mostly lowering) of plasma albumin concentrations as a result of modifications in the rate of synthesis, catabolic rate, or the distribution between the extravascular and intravascular spaces. Some of the diseases that lower plasma albumin concentrations are summarized in Table 24.

In acute injury or disease, reduced plasma levels of albumin and increased γ-globulin levels in plasma are thought to be the normal host reaction to injury or infection rather than being a response to any specific disease. Burns, myocardial infarction, and surgery are followed by decreased plasma albumin levels. Acute febrile infections such as pneumonia, tonsillitis, scarlet fever and rheumatic fever, also cause a decrease in albumin levels and an elevation in γ-globulin levels.

Surgical procedures can produce a prolonged negative protein balance, largely owing to loss of proteins into the traumatized area and the increased protein demands of the regenerating tissues.

Burns cause the most pronounced decrease in plasma albumin. This occurs, firstly, because the skin contains the largest fraction of extravascular albumin and destruction

Table 24

Diseases which lower plasma albumin

Small change	Larger change
Bone fractures	Liver disease
Myocardial infarction	Renal disease
Surgery	Burns
Acute infection	
Pregnancy	
Neoplastic disease	
Gastro-intestinal disease	
Chronic fibrosis	
Bedrest	
Smoking	
Aging	

of an appreciable portion of skin results in a significant loss of interstitial albumin. Secondly, capillary permeability is increased after extensive burns and this increased permeability causes albumin depletion from the plasma compartment and marked extracellular pooling of albumin, especially in the initial phase of burn.

Neoplastic diseases are associated with lower serum albumin. As the disease becomes more disseminated, the albumin concentration progressively decreases due to diminished albumin synthesis.

Liver diseases are the most complex and prevalent of the chronic disorders afflicting man. The liver synthesizes most plasma proteins and hepatic impairment, especially chronic disease, can result in diminished levels in the body.

Marked elimination of albumin occurs in diseases affecting the kidney. Where the glomeruli and tubules are both affected in chronic renal disease, excessive protein filtration may lead to increased loss of albumin from the body. Nephrosis, in particular, is associated with severe hypoalbuminaemia.

It has been established that the elderly are particularly prone to drug toxicity (Hurwitz, 1969). This implies that there may be some alteration in drug handling with aging. A variety of factors may operate to alter the response of an elderly patient to a drug. Changes with plasma protein concentrations occur with advancing age. The pattern generally found has been a fall in albumin level with a rise in γ-globulin concentration as age increases. The most important cause for these changes appears to be related to decreased mobility in elderly people.

6.1.2.2 Effect of diseases

Patients with impaired renal function have a high incidence of adverse drug reactions. Such drug reactions were originally thought to be mainly the result of decreased excretion of the drug by the kidneys leading to the accumulation of the drug in the body. However, it is well recognized that the binding capacity of many drugs is less in ureamic patients than in normal subjects. Most of the drugs that show such abnormal binding are organic acids.

Reduced plasma protein binding of drugs is expected to result in a greater tissue distribution, a higher apparent volume of distribution, and a lower plasma concentration of the drug. Thus, ureamic patients may respond to drug therapy at relatively lower total plasma concentrations than non-ureamic patients.

Plasma concentrations of free fatty acids increase in patients with renal failure as a result of mobilization of adipose tissue (Losowsky and Kenward, 1968). The affinity of free fatty acids for albumin is greater than that of most drugs, and, in sufficient quantity, free fatty acids could be expected to compete for some of the drug binding sites on albumin. Rudman, Bixler and Del Rio (1971) have found that in the concentration range normally seen in acute renal failure, free fatty acids have a general inhibitory effect on the binding capacity of serum albumin with eight different drugs.

The reduction in plasma protein levels in disease states reduces the binding of drugs thus allowing more free drug to become available for distribution throughout the body tissues, thus enhancing, at least transiently, the effect of that

drug, or causing more drug to become available for metabolism. This is of particular importance for highly bound drugs (90% or more); the effects for less extensively bound drugs would be less marked.

The most critical factor in the development of gentamicin ototoxicity has been reported to be impaired renal function (Jackson and Arcieri, 1971). This toxicity must be result of high concentrations of gentamicin in serum due to reduction in excretion. The critical level in the production of ototoxicity is unknown.

In uraemic patients the binding capacity of serum albumin and drugs is decreased. It has been suggested that this decrease may be the result of the increased non-esterified fatty acids interacting with the albumin molecule and reducing the number of sites available for drug binding (Dromgoole, 1973). The clinical significance of this is that binding of gentamicin may be reduced in ureamia and in patients after kidney transplantation. This would result in increased plasma levels of 'free' gentamicin and possibly be a contributing factor to the high incidence of ototoxicity found in these patients. Drug dosage modification to obtain plasma therapeutic levels would appear to be most beneficial in patients with the abnormalities of gentamicin excretion.

Previous studies on the extent of gentamicin binding to serum proteins are conflicting. The results are difficult to evaluate because of the different techniques employed. The present study was undertaken to examine the binding of gentamicin. In the first instance, the binding of the drug was examined to resolve the variations cited. Once this had been

established, then the protein resposible for the binding of gentamicin was determined by the electrophoretic method. The concentration of gentamicin used was that commonly found in blood during therapy (5 μg/ml). Physiological concentrations of the divalent cations, magnesium and calcium, were used.

6.2 Methods and Materials

6.2.1 Materials

^{3}H-labelled gentamicin sulphate was supplied by the Radiochemical Centre, Amersham, England. The sample supplied had a specific activity of 514 mCi/mmol. Unlabelled gentamicin sulphate was dissolved in Sorensen's phosphate buffer (Sørensen, 1909) at pH 7.4 to give a concentration of 5 µg/ml and labelled gentamicin was added to give an activity of 11526 disintegrations per minute per millilitre of solution.

6.2.2 Serum

Pooled human serum was prepared from clotted whole blood obtained from fifteen healthy volunteers who had not recently received antimicrobial agents or other drugs. It was stored at 4^{o}C and used as required. The amount of protein present was found to be 7.9 g/100 ml as determined by the Biuret method (Reinhold, 1953).

6.2.3 Scintillation Fluor

5 g 2,5-diphenyloxazole (PPO) supplied by Hopkin and Williams, Chadwell Heath, Essex, England.
0.1 g 1,4 di(2-(5-phenyloxazolyl))benzene (POPOP) supplied by Hopkin and Williams, Chadwell Heath, Essex, England.

Made up to 1 litre with Toluene (Hopkin and Williams). Two parts of the scintillant was mixed to every part of Triton X-100 surfactant (scintillation grade). 500 ul of the sample was added to 5 ml of the scintillation solvent in glass scintillation counting vials. The vials were counted using the liquid scintillation spectrometry for 10 minutes each. Counting was corrected for quenching by the internal standardization method.

6.2.4 Internal standardization

Sample was counted (A counts/min). It was removed from the counter and a small amount of standard material of known disintegrations per minute (B d/min) was added. The sample was then recounted (C counts/min). The counting efficiency of the sample was then calculated from:

$$((C - A) / B) \times 100)$$

Then the corrected sample disintegrations per minute $= A \times \frac{100}{\% \text{ efficiency}}$

6.2.5 Gel-filtration

6.2.5.1 Method

A 1.1 x 60 cm column of coarse sephadex G-25 was equilibrated with 1.1×10^{-5}M (5 µg/ml) solution of ^{3}H-labelled gentamicin in 0.1M Sørensen's phosphate buffer (Sørensen, 1909) at pH 7.4 by passing through the column a volume of solution equivalent to three times the void volume of the column (void volume = 18.4 ml). 0.14 ml of serum was mixed with 5 ml of the solution used to equilibrate the column. After 30 min at room temperature, 1 ml of the solution was transferred to the top of the sephadex column, and 10 min later, the protein solution was allowed to run into the column. The reservoir was reconnected to the column and the elution with the solution used to equilibrate the column was allowed to proceed. The flow rate of the column was 6ml/h. By means of a fraction collector, the effluent solution was collected in serial fractions of 0.5 ml.

After the run was over, the amount of gentamicin present in each fraction was determined by counting the amount of radioactivity present in each sample using liquid scintillation spectrometry.

6.2.5.2 Calculations

The amount of gentamicin bound to the serum proteins during the run was determined from the area of the trough in the elution pattern (Fairclough and Fruton, 1966). The difference between the base-line concentration of each fraction constituting the trough was determined and used in the equation:

$$\text{apparent umoles bound} = \frac{\sum i\ (\Delta Ai \times mli)}{\text{molecular weight}}$$

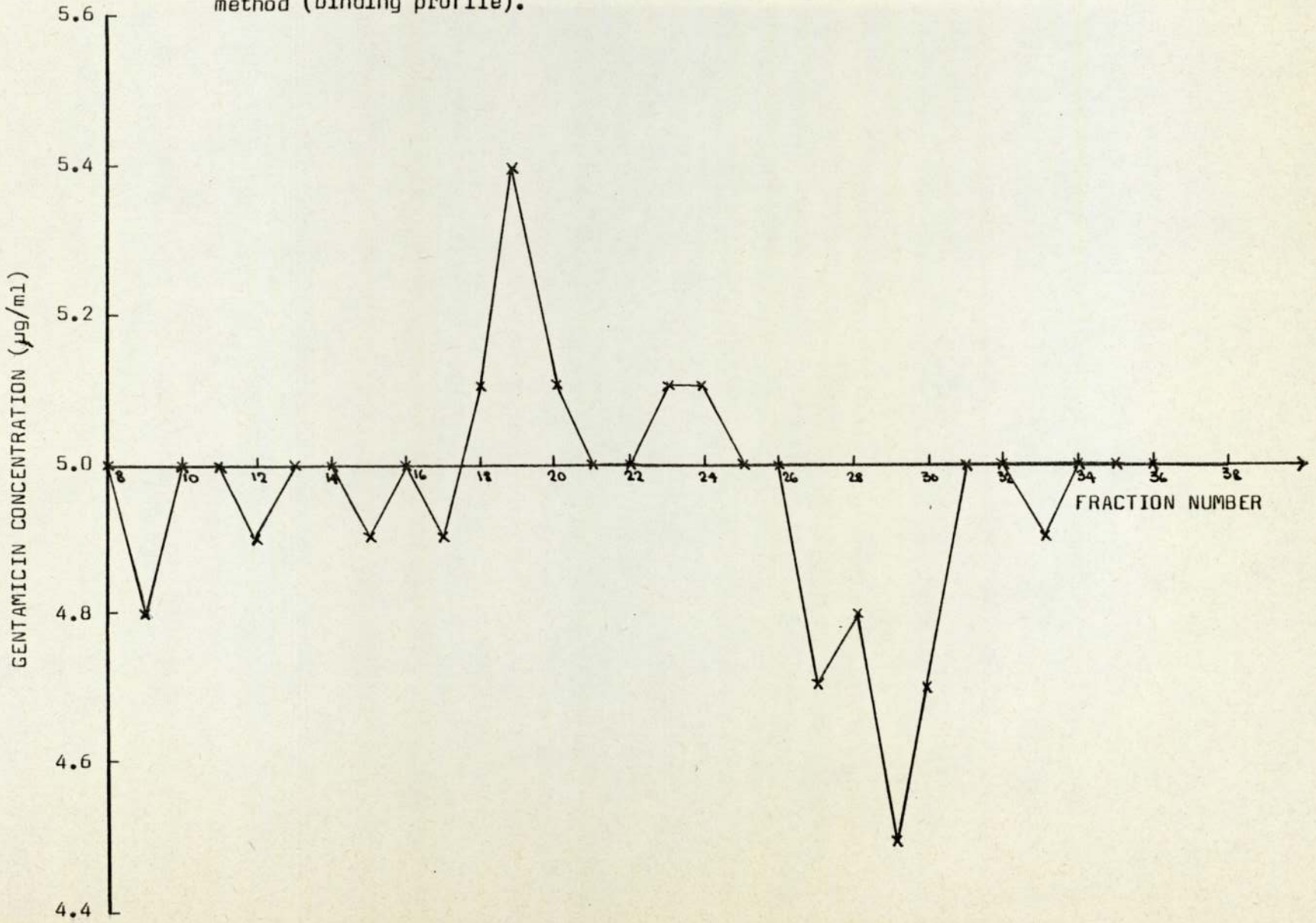
where ΔAi is the difference between the base-line concentration value and that of fraction i of the trough;

mli is the volume of fraction i.

6.2.5.3 Results

Figure 21 shows a representative elution pattern for the gentamicin-serum system obtained by the determination of the radioactivity present in individual samples of the effluent solution. The results represent the mean values of at least three determinations. The amount of drug bound was calculated to be 0.24 µmoles which was 26% of gentamicin.

Fig. 21 Binding of gentamicin to serum proteins as demonstrated by the gel-filtration method (binding profile).



6.2.6 Continuous Ultrafiltration (diafiltration)

6.2.6.1 Method

5 µg/ml of ^{3}H-labelled gentamicin solution in Sørensen's phosphate buffer (Sørensen, 1909) at pH 7.4 was placed in the reservoir (Fig. 22). This reservoir solution was then used in two separate continuous ultrafiltration experiments. In the first, only the buffer solution was in the Amicon ultrafiltration cell (capacity 10 ml), in the second, protein solution was in the cell. In both cases, ultrafiltration was initiated until the concentration of the effluent reached the concentration of the initial drug concentration. Graphs were plotted and the area within the two curves represented the amount of drug bound to the protein (Fig. 23).

Having filled the tubing and selector valves with the gentamicin solution, the flow was stopped by the manifold switch and the ultrafiltration cell connected by selector switch to the nitrogen gas cylinder, allowing equal pressurization of the whole system. This minimized the volume changes in the cell. The selector valve was then changed to connect the reservoir to the cell, the manifold switch was simultaneously opened, and ultrafiltration commenced. Ultrafiltration was carried out at 25 p.s.i. pressure using the Diaflo membrane PM-10 (Amicon Corp., Lexington, Mass.) which restricted the passage of macromolecules in excess of 10,000 molecular weight. Ultrafiltration cell (fitted with Teflon-coated stirrer) was made firm by clamping, and a magnetic stirring table was placed below to effect mixing. Agitation was needed to maintain uniform bulk composition in the cell to prevent adhesion of high molecular weight species

Fig. 22 Experimental set-up for the continuous ultrafiltration apparatus.

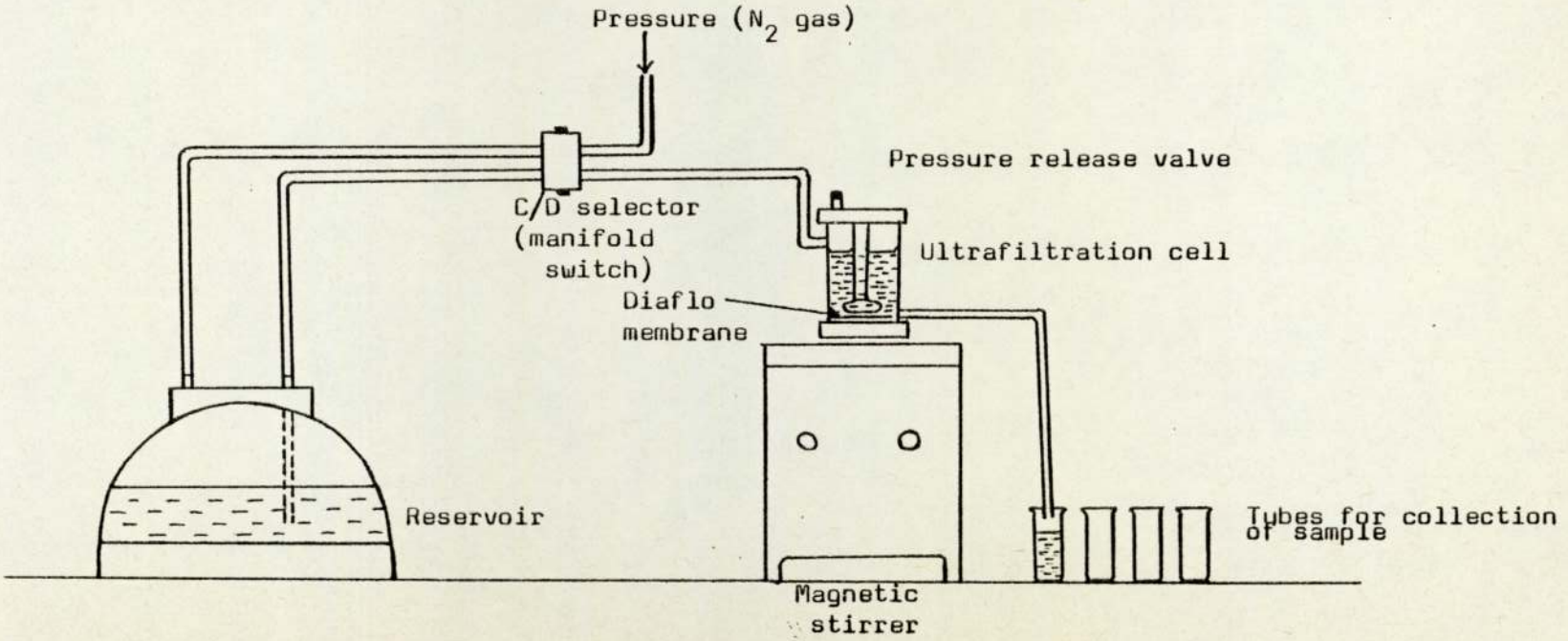
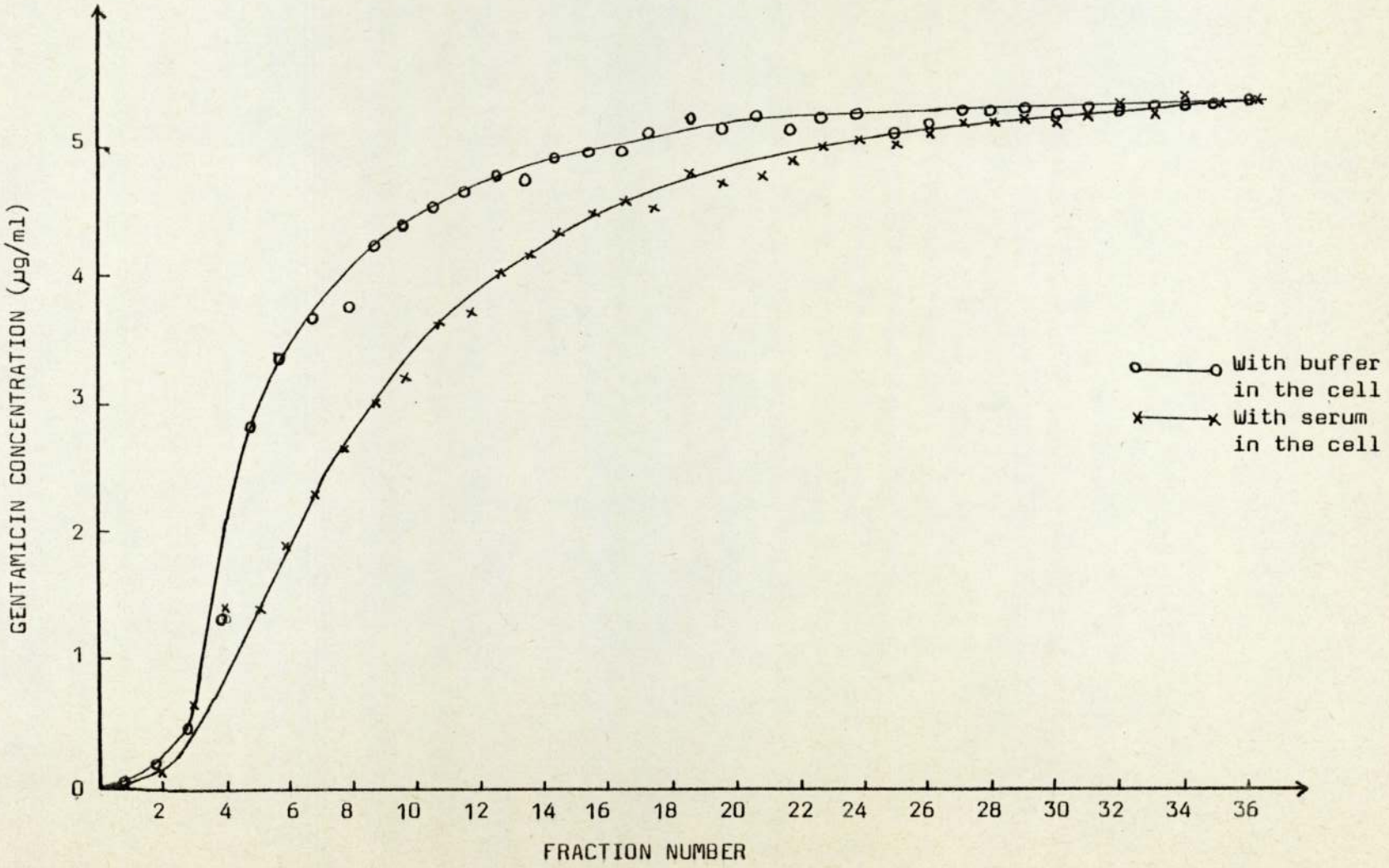


Fig. 23 Elution curves for the continuous ultrafiltration experiment.



against the membrane surface. The ultrafiltrate which formed at the rate of 11 ml/h, was collected in 1.5 ml volumes in clean dry tubes using an LKB Ultrofac fraction collector with a drop counter to minimise cross-contamination of fractions. As the ultrafiltrate was discharged from the cell via the outlet port, fluid was replaced from the reservoir. In essence, a series of separate ultrafiltration experiments at constant pressure but increasing concentration of gentamicin solution were performed in sequence. However, in each collection period, concentration of total, free and bound gentamicin could be precisely calculated or measured. The amount of radioactivity present in each tube was determined using liquid scintillation spectrometry.

6.2.6.2 Calculations

Let vi = volume in litres of the ith ultrafiltrate collected.

V = volume of solvent in the cell in litres.

R = Gentamicin concentration in the reservoir (moles per litre).

(M)i = Gentamicin concentration in the ith collection (moles per litre).

Since it is a closed system, the volume of solution entering the cell in the ith period exactly equals the volume leaving (vi);

Therefore:

Moles into cell = vi(R)

Moles out of cell = vi(M)i

Moles retained = vi(R) - vi(M)i

At the end of the ith period, the:

Increase in total concentration in cell during ith period $= \frac{1}{V}\left[vi(R) - vi(M)i\right]$

At the end of the nth period:

$$\text{Total moles/litre in cell} = \frac{1}{V} \sum_{i=1}^{n} \left[vi(R) - vi(M)i\right]$$

During the nth period, the:

Gentamicin concentration in ultrafiltrate collected = moles/litre of gentamicin in cell

$$= (M)_n$$

$$\text{Concentration of bound drug} = \frac{1}{V} \sum_{i=1}^{n} \left[vi(R) - vi(M)i\right] - (M)_n$$

6.2.6.3 Results

The curves obtained, using the ultrafiltration cell first with pure buffer solution to obtain a control drug 'wash-in' curve, and then with serum solution to obtain a binding 'wash-in' curve are shown in Figure 23. The binding curve for the concentration of free gentamicin versus total concentration is shown in Figure 24. The broken line in Figure 24 shows the curve for no binding. The binding detected for gentamicin to serum proteins was found to be 24% which was not strong.

From this experiment, Scatchard plot (1949) was constructed, analysis of which gave the number of binding sites and their respective association constants (Fig. 25).

The Scatchard method of plotting of protein-bound drug data is based on the Law of Mass Action. In discussing the relevance of drug-protein interactions, both the affinity or strength of binding (usually expressed as an apparent association constant k) and the capacity of the protein sites (n) should be considered. For completely reversible associations the interaction of the drug (D) with the unoccupied binding

Fig. 24 Binding isotherm of free gentamicin versus total (bound and unbound) gentamicin for binding gentamicin to human serum. The dotted line represents the curve for no binding.

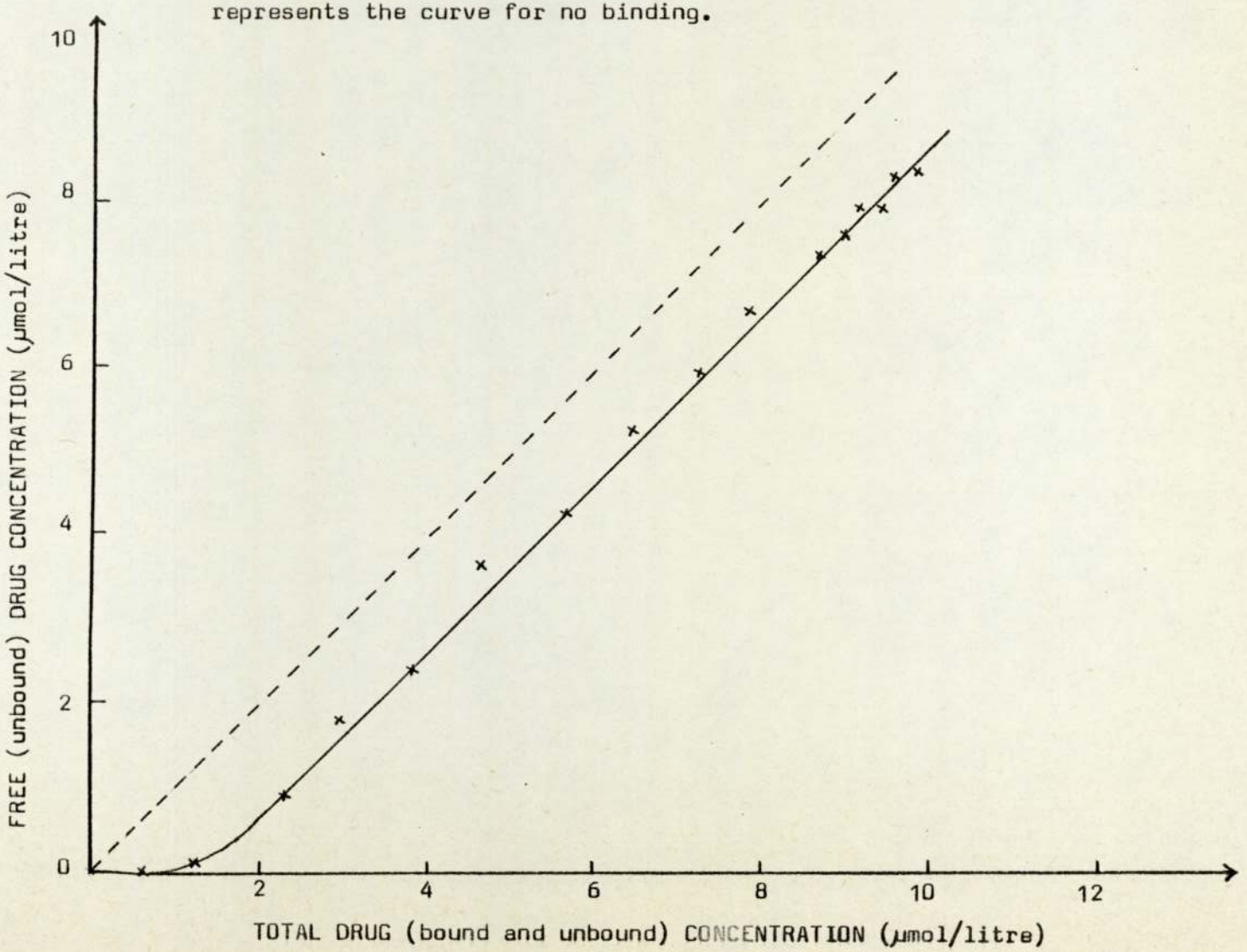
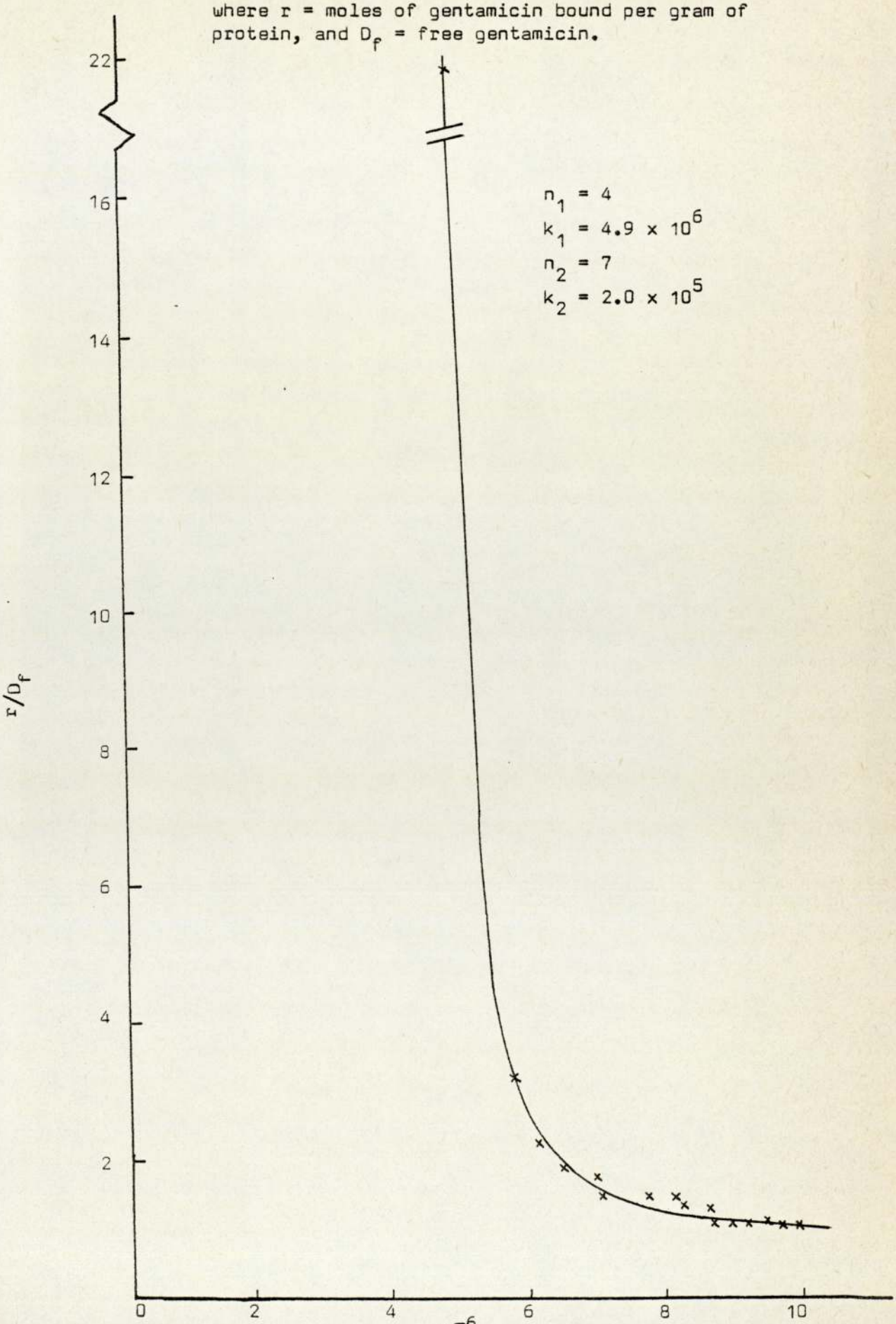


Fig. 25 Scatchard plot of gentamicin–serum proteins interaction, where r = moles of gentamicin bound per gram of protein, and D_f = free gentamicin.



sites of a protein (P) may be considered to obey the Mass Law of Action:

$$D_f + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DP$$

where DP is the protein-drug complex and k_1 and k_2 are the rate constants of the forward and reverse reactions.

At equilibrium therefore

$$\frac{(D_f)\,(P)}{(DP)} = \frac{k_1}{k_2} = K \qquad \text{equation (1)}$$

where D_f is the molar concentration of the free (unbound) drug,

P is the molar concentration of protein,

DP is the molar concentration of the drug-protein complex, and

K is the equilibrium or dissociation constant. The reciprocal of this latter term (k), the association constant, is the measure of the affinity of the drug for the protein.

This is obviously the simplest situation in which a homogenous binding protein with a single binding site for the drug is involved. In cases where several binding sites or proteins are concerned, a succession of such equilibria are required to describe the overall interaction. Considering the general case of the binding of a protein which contains n binding sites (independent), each exhibiting a similar affinity k for the drug, then n(P) will be the total concentration of binding sites for the drug

$$\text{Therfore,} \qquad n(P) = (DP) + (P_f)$$

$$\text{i.e.} \qquad (P_f) = n(P) - (DP) \qquad \text{equation (2)}$$

where (P_f) is the molar concentration of the unbound protein

Substitution of equation (2) into (1) yields:

$$\frac{(n(P) - (DP))\,(D_f)}{(DP)} = \frac{1}{k}$$

which by rearrangement gives:

$$n(P)(D_f) = (DP)\left(\frac{1}{k} + (D_f)\right) \qquad \text{equation (3)}$$

Now, if r = moles of drug bound per mole of protein

$$r = \frac{(DP)}{(P)}$$

Rearrangement of equation (3) gives:

$$r = \frac{nk\,(D_f)}{1 + k(D_f)} \qquad \text{equation (4)}$$

Equation (4) can be rearranged to yield:

$$\frac{r}{(D_f)} = nk - rk \qquad \text{equation (5)}$$

which is the basis for the Scatchard plot in which $\frac{r}{(D_f)}$ is plotted as a function of r; extrapolation to abscissa and ordinate allows estimation of n and nk. Curvature of the plots is usually indicative of the existence of more than one class of binding sites.

When the data obtained by the continuous ultrafiltration method was plotted as a Scatchard plot, the plot was curved. This indicated that there were either more than one class of binding site on the protein or there were more than one species of binding protein.

6.2.7 Frontal Analysis Chromatography

6.2.7.1 Method

Sephadex G-25 (fine grade) was allowed to swell in 0.1M Sørensen's phosphate buffer (Sørensen, 1909) at pH 7.4 at room temperature for 20 h. This was packed into a glass column (internal diameter 5 mm) to give a column of 16 cm long. This was equilibrated for 4 h with the buffer supplied at 15 ml/h by a peristaltic pump. Sample solution (12 ml) containing 1 ml of serum and 10 ml of ^{3}H-labelled gentamicin (5 µg/ml) was introduced through the pump and 10 drop fractions which were approximately 0.5 ml were collected during sample introduction and subsequent elution with 15 ml of buffer. Alternate fractions were assayed for protein and gentamicin. Protein was estimated by the Biuret method (Reinhold, 1953) using bovine serum albumin as the standard. ^{3}H-labelled gentamicin was counted as before using liquid scintillation spectrometry.

6.2.7.2 Results

Figure 27 shows the elution patterns for the gentamicin and the protein. The standard graph for bovine serum albumin was plotted to calculate the protein concentrations (Fig. 26). Two distinct leading and trailing boundaries were noticed (Fig. 27) for the gentamicin zone. From the concentrations of gentamicin at the leading and trailing boundaries in the elution pattern, the fraction of the drug bound was calculated to be 26% (Cooper and Wood, 1968).

Fig. 26 Calibration graph for the estimation of protein.

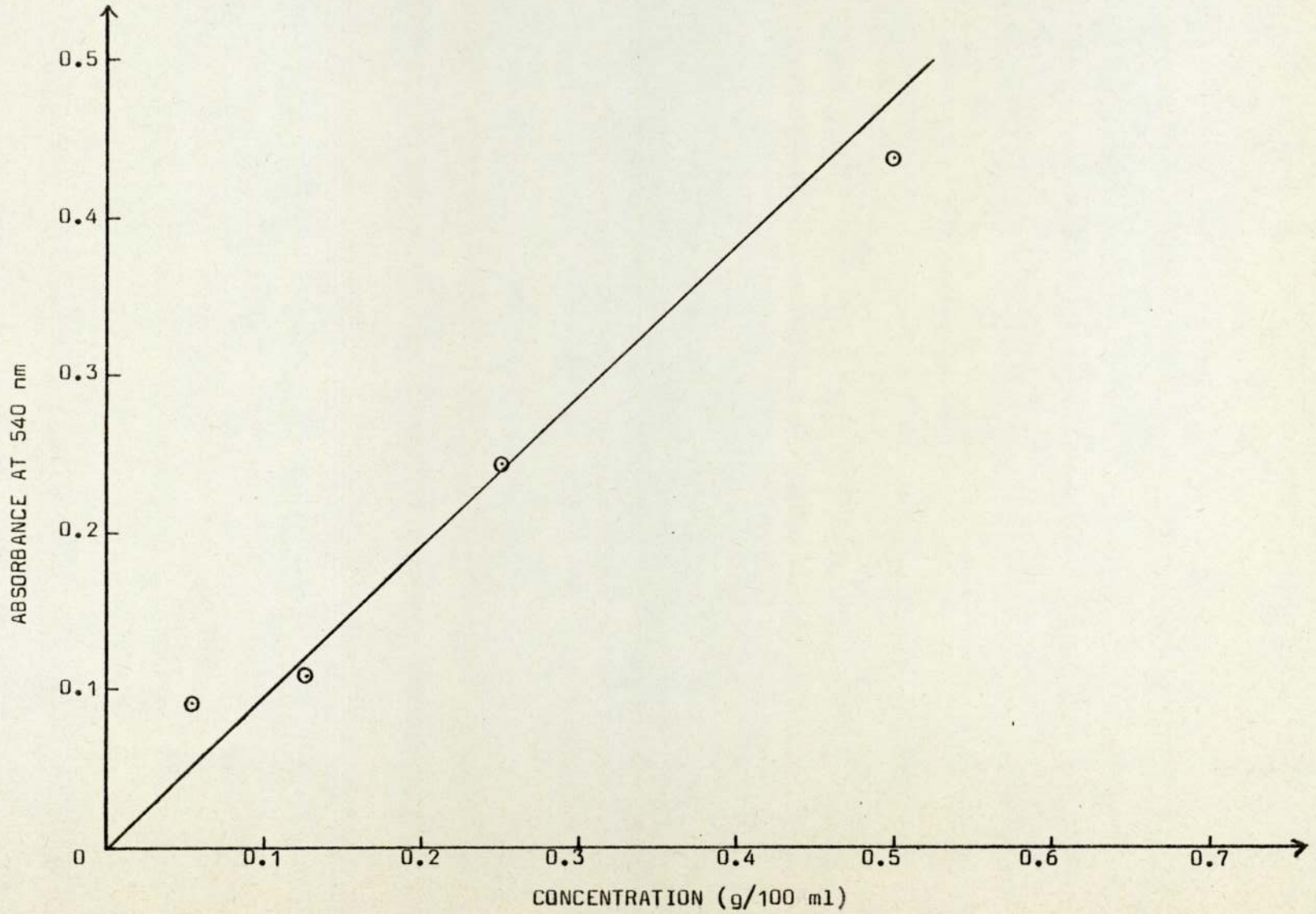
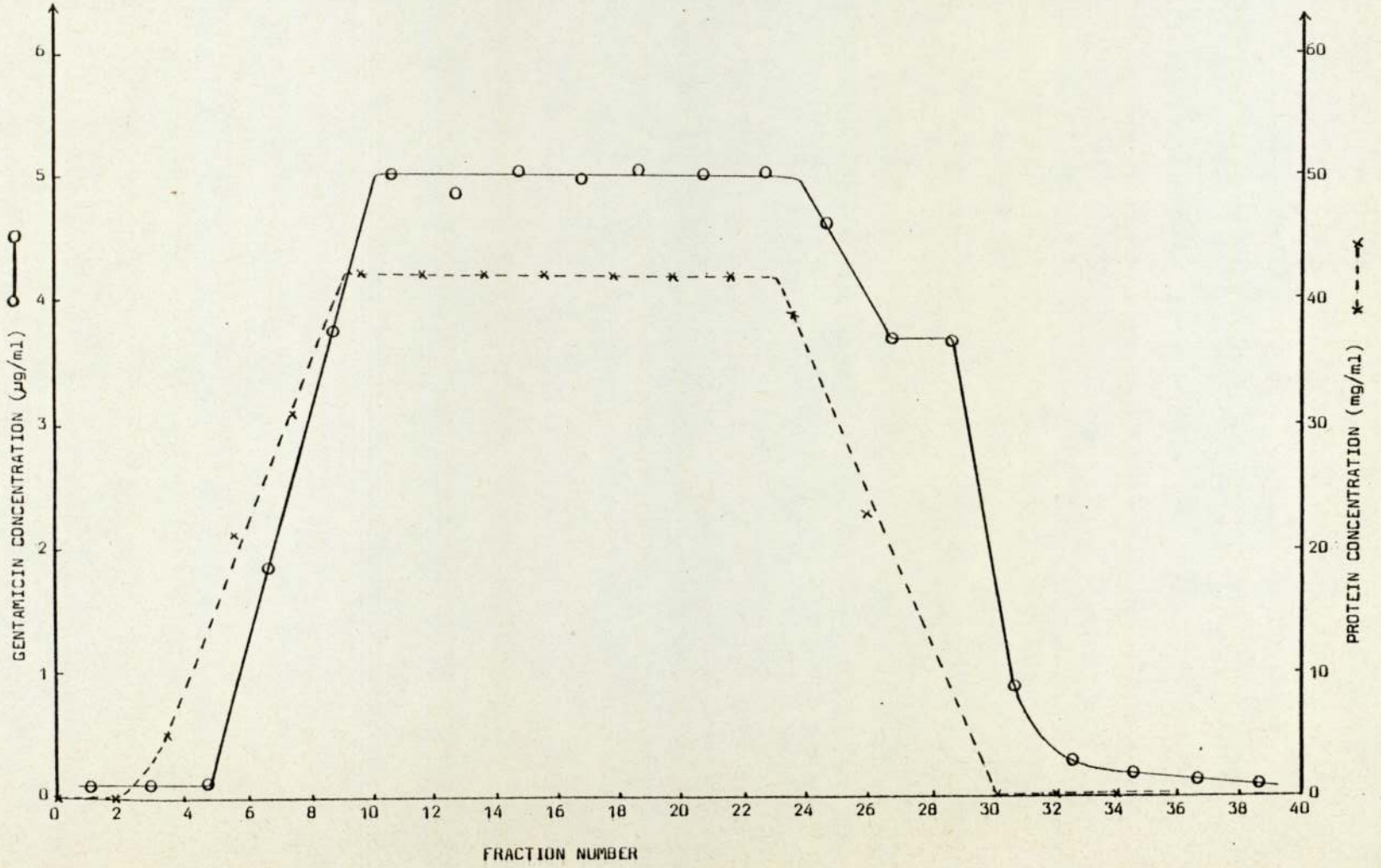


Fig. 27 Elution patterns for protein and gentamicin obtained by the Frontal analysis method.



6.2.8 Electrophoresis

6.2.8.1 Methods and Materials

Electrophoresis was applied not only to determine which serum protein was responsible for the binding of gentamicin but also to calculate the amount of drug bound to this protein or proteins.

6.2.8.2 Materials

'Shandon' electrophoresis tank and power pack.

'Shandon' Celagram cellulose acetate strips, size 25 x 60 cm.

5 µl micropipette.

Methanol.

Buffer

Barbitone	1.3 g
Sodium barbitone	9.1 g

These were dissolved in glass distilled water and made up to 1 litre to give a buffer of pH 8.6 with an ionic strength 0.05. 5 ml of 5% thymol in isopropyl alcohol was added to the buffer as a preservative.

Fixative-dye solution

2 g of Ponceau S dye and 30 g of trichloroacetic acid were dissolved in glass distilled water and made up to 1 litre.

Rinse solution

5% (v/v) acetic acid in distilled water.

6.2.8.3 Method

The chamber was filled with buffer to the required level and covered to equilibrate. A small amount of the buffer was placed in a shallow tray and the cellulose acetate strips were floated on the buffer to allow the buffer to be drawn up

evenly through the pores without trapping air bubbles. The strips were then completely immersed in the buffer by agitating the tray. The acetate strips were then removed from the buffer bath and gently blotted between two strips of clean filter paper for about three seconds. 0.1 ml of serum was equilibrated with 2.5 ml of ^{3}H-labelled gentamicin (5 µg/ml) for 4 h. 5 µl of this was applied to each strip by a gentle stroking motion in a straight narrow band taking care to avoid bubble formation. Normal serum which did not contain radioactive gentamicin was used as well. The starting position for the line of application of sample was about 1 cm from the centre towards the cathode side. The cover was then placed tightly on the chamber and the power supply was turned on. About 30 seconds was allowed to warm up and the voltage was adjusted to 220 V. The experiment was left to run for one hour and thirty minutes to allow a good separation of the proteins.

At the end of this time, the power supply was turned off and the strips were removed from the chamber and floated on 100 ml of the fixative-dye solution with the serum side up. As soon as the protein bands were stained with the dye, the strips were immersed completely and allowed to stand for about 10 min. After the dyeing was complete, the strips were transferred to the rinse solution and agitated gently. Fresh rinse solution was replaced after 2 min. Three washes were suffice to obtain a completely white background. Washing was then completed in methanol for 5 min and the strips were dried.

Strips containing tritiated gentamicin-serum were not fixed or washed but were cut into equal bands corresponding to the protein zones, obtained on the stained strips, by

placing the two strips alongside each other. These bands were counted by liquid scintillation spectroscopy. Correction for background counts was made by counting the clear piece of cellulose strip of equal length.

6.2.8.4 Results

Five zones were recognized at pH 8.6. They corresponded to albumin, α_1, α_2, β and γ-globulin. Results indicated that 31% of the gentamicin was associated with the albumin fraction of the serum. No gentamicin was detected in any of the other zones.

6.3 Discussion and Conclusions

Under the conditions used in these experiments, the binding of gentamicin to serum proteins ranged from 24 to 31% depending on the method employed. The results were tabulated and are shown in Table 25. The protein responsible for this binding was found to be albumin as shown by the electrophoresis of tritiated gentamicin. It had two distinct classes responsible for the binding of gentamicin and the number of binding sites (n) were found to be $n_1 = 4$ and $n_2 = 7$. Their respective association constants were $k_1 = 4.9 \times 10^6$ and $k_2 = 2 \times 10^5$.

Results showed that as long as the conditions of the experiment were controlled precisely, one can expect to obtain similar results with any of the methods employed in this study. Of the four methods studied, continuous ultrafiltration method was the only method which made it possible to construct a binding curve over a wide range of gentamicin concentrations. From this curve, it was possible to calculate the number of drug binding sites per mole of protein and the affinity of the drug for these sites. However, this method together with the gel-filtration method of Hummel and Dryer (1962) required large quantities of the drug solution. Cooper and Wood (1968) method of frontal-analysis chromatography overcame this problem and was faster than all the methods employed.

These results were in agreement with the results of the studies performed by some other investigators (Bulger, Sidell and Kirby, 1963; Riff and Jackson, 1971 and Myers et al., 1978).

Bulger and associates (1963), using an ultrafiltration technique, reported that 30% of gentamicin was bound to serum proteins. Methodological details such as drug concentration,

Table 25

Comparison of the results obtained for the binding of gentamicin by employing different methods

Method	Percentage of gentamicin bound
Gel-filtration (Hummel and Dryer, 1962)	26.0
Continuous Ultrafiltration (Blatt, Robinson and Bixler, 1968)	24.0
Frontal Analysis Chromatography (Cooper and Wood, 1968)	26.7
Electrophoresis	31.0

preparation of dialysis tubing and binding of the drug to the dialysis tubing were not discussed. Utilizing a different approach, Riff and Jackson (1971) studied the distribution of gentamicin (100 μg/ml) in whole blood and plasma to which autologous erythrocytes were added. After centrifugation, which removed the erythrocytes and 10% of the radioactivity, plasma proteins were precipitated by addition of trichloroacetic acid, which removed another 30% of the radioactivity. These results were interpreted as demonstrating 30% serum protein binding of gentamicin, however, no details were given about the effect of trichloroacetic acid on this basic antibiotic itself. Other factors to be considered are the possible effects of the trichloroacetic acid on the protein binding sites and the tertiary structure of plasma proteins. The binding affinity may be altered by this acid. Also the high concentration of gentamicin used was not in the usual clinical range.

Gordon, Regamey and Kirby (1972) found no significant gentamicin binding to serum proteins under controlled conditions of physiological pH and temperature by means of an ultrafiltration technique. The concentration of gentamicin used was similar to the present study but they used an agar well method for the determination of the drug concentrations. Most recently, Myers et al. (1978) reported that 27% of gentamicin was bound to serum proteins with no calcium and magnesium present. They demonstrated that under physiological conditions in the presence of divalent cations, the drug binding decreased to 20%. Even when divalent cation concentrations were raised 4-fold, binding was not reduced below 17%.

In the present study, the effect of the divalent cations were not studied since it was not extensive in the first place and also the results were in good agreement with Myers's study when no cations were present.

The observations that renal clearance of aminoglycosides are similar to the glomerular filtration rate suggests that the amount of binding demonstrated in this study is not physiologically important. Nevertheless, the fact that gentamicin is bound to serum proteins to a measurable degree may be important in drug transport from peritubular blood into the renal tubular cell. The kinetics of gentamicin uptake into renal tissue are not well understood. Cortical concentrations of gentamicin twenty times those of concomitant serum levels have been reported (Luft and Kleit, 1974). They stated that accumulation of aminoglycosides is predominantly cortical, that is, in an area consisting largely of proximal tubules. The tubular cells would then be susceptible to aminoglycoside toxicity as a result of this acculmulation. With gentamicin, the pharmacokinetic responses of individual patients to standard regimens are notoriously unpredictable (Riff and Jackson, 1971). The possibility that significant binding of gentamicin to serum or tissue proteins might occur under pathological conditions in man and might help to determine variations in the pharmacokinetics or in the antibacterial effectiveness of gentamicin in individual patients should therefore be considered and deserves investigation in appropriate clinical conditions.

SECTION 7

THE INFLUENCE OF DIFFERENT FIXATIVES ON THE ULTRASTRUCTURE OF THE ORGAN OF CORTI

7.1 Introduction

Electronmicroscopical (EM) techniques are valuable for the study of pathological changes induced by drugs or noise trauma in the organ of Corti. In such studies the preservation of ultrastructural detail is of critical consequence in the interpretation of both normal and pathological cellular processes.

The interpretation of the results of studies by Harpur and Bridges (1979) on the evaluation of the EM methods for the investigation of the gentamicin-damaged guinea-pig organ of Corti was complicated by the appearance of common ultrastructural defects in both the control and drug-injected animals. These defects included mitochondrial swelling with separated or disintegrated cristae, cytoplasmic vacuolation and shrinkage. Occasionally, the bulging of the lateral membranes was also noticed especially in the middle turns of the organ of Corti. Thus, it was difficult to attribute minor intracellular changes to drug administration.

Other than the processing of the tissue, the elapse of time between death and primary fixation is an obvious source of ultrastructural damage. Most inner ear structures are quite sensitive to the amount of time elapsed from the animal's death to the application of the fixative. Wersäll, Kimura and Lundquist (1965) described the post-mortem changes in the guinea-pig organ of Corti at the time intervals of 5, 10, 15, 30, 45 minutes, 1 h, 3 h and 6 h post-mortem. One of the first noticeable changes observed were swelling of the mitochondria

in the nerve endings and in the lower part of the sensory hair cells. They measured the smallest diameter of 50 mitochondria from the large nerve endings and 50 mitochondria from the adjacent part of the outer hair cells from the first row of the basal turn. The difference between the mean values of the control animals, killed under anaesthesia and the cochleas fixed by the in vivo method, were compared with the mean values of the other experimental groups using the Student's t-test. The outer hair cell and the nerve ending mitochondria were analysed separately using a highly significant level ($P < 0.001$). Wersäll et al. (1965) demonstrated that 15 minutes after post-mortem, statistically significant ultrastructural changes had occurred within the organ of Corti.

Minor changes in preparative technique may also influence the fine structure of cells. Fixation is the first step in the preparation of biological specimens for electron microscopy and some fixatives appear to selectively alter cellular details. The aim of fixation is to convert the labile structural organization of the living material into a stable state so that it can be subjected to the necessary manipulations of morphological investigation without further change. With some fixatives obvious disruption of fine structures such as discontinuities in membranes, transformation of tubules into chains of vesicles have been observed (Glauert, 1975). Some of these effects vary with the nature of the specimens as well as the fixative and in consequence a number of criteria influence the choice of a fixative for a particular specimen. Aminoglycoside antibiotics are known to be toxic to the tissues of the inner ear (Kohonen, 1965; Lundquist and Wersäll, 1967). The

preparation of the organ of Corti presents peculiar problems and requires special techniques. The preparation of the organ of Corti is more complicated than that routinely used for other organs for several reasons. The inner ear is:

1. small in size.
2. very delicate in structure and, for this reason, it is encased in the temporal bone; consequently it is not readily accessible.
3. made up of several very different tissues, i.e. epithelial and connective tissue, nerve and bone.

and

4. contains special fluids, the endolymph and perilymph and the sensory structures within the ear are located further than the nearest blood capillary than most other cells in the body (Iurato, 1976).

Glutaraldehyde fixation followed by the 'post-fixation' with osmium tetroxide has been extensively used. In the present study fixation by local perfusion of the cochlea was compared with vascular perfusion. The effect of solutions of glutaraldehyde at varying concentrations and osmolarity on the neuroepithelium of the organ of Corti was also investigated. Lastly, the influence of calcium ions in the fixatives were studied.

7.2 Methods and Materials

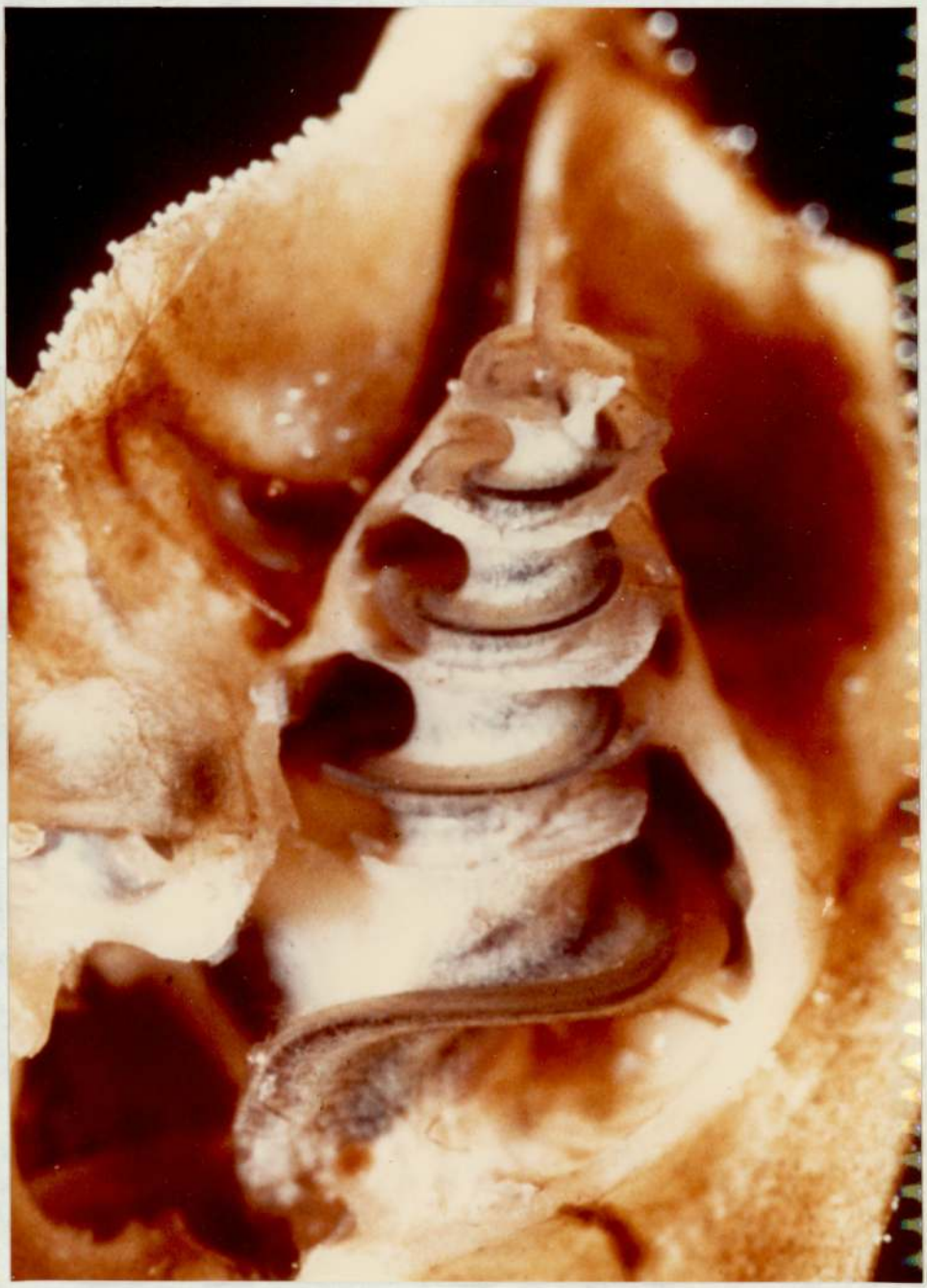
Young healthy male albino guinea-pigs weighing 200 - 250 g were used. Only animals exhibiting a good Preyer (pinna) reflex were used and no animal showed signs of otitis media at post-mortem.

7.2.1 Fixation and preparation of the cochlea

The guinea-pigs were killed by cervical dislocation followed by decapitation. As quickly as possible the skull was split along the sagital plane and the ventral surfaces of the auditory bullae were exposed. Temporal bones were then removed and opened taking care not to damage the cochlea. Openings were made in the round window and the apex and the cochleas were then fixed successively either by perfusion or immersion with phosphate buffered (Millonig, 1961) glutaraldehyde at varying concentrations for 2 h ($4^{o}C$) and 1% osmium tetroxide for 2 h ($4^{o}C$) separated by a 24 h wash in buffer with several changes. Glutaraldehyde was introduced as rapidly as possible following decapitation (not later than 4 min) to safeguard against ultrastructural post-mortem changes (Wersäll et al., 1965). After fixation, the cochleas were transferred to 50% alcohol for 15 min and then to 70% alcohol.

In 70% alcohol, the dissection was carried out under a binocular dissection microscope. Using sharpened dental instruments, the bony capsule was broken away over a considerable area and the spiral ligament together with stria vascularis were removed, thus uncovering the coils of the cochlea and the organ of Corti (Fig. 28). With small watchmaker's tweezers, the entire cochlea was dissected systematically in half turns starting at the base and working up to the apex. Specimens

Fig. 28 Right cochlea of the guinea-pig after the removal of the otic capsule (x 19)



were then dehydrated in graded alcohol solutions right through to 100% alcohol. A modified technique of dehydration was used whereby the specimens were taken through small increases of alcohol concentrations to avoid artefacts produced by dehydration (Mercer and Birbeck, 1972).

7.2.2 Dehydration schedule

80% alcohol in water	15 min Room Temperature (rt)
90% alcohol in water	15 min rt
95% alcohol in water	15 min rt
100% alcohol	15 min rt
100% alcohol	15 min rt

The specimens were embedded in low-viscosity Spurr resin (Spurr, 1969) as follows:

Propylene oxide	15 min rt
1:1 mixture of propylene oxide and Spurr resin	15 min rt
Spurr resin	overnight

The specimens were then transferred to fresh Spurr resin for embedding and polymerised at 60^{o}C overnight. Glass distilled water was used to prepare all solutions.

Using an LKB ultrotome and glass knives, 1 µm sections were prepared for light microscopy and stained with toluidine blue and ultrathin sections (60 - 90 nm) for electron microscopy were cut and stained with uranyl acetate and lead citrate (Reynolds, 1963) using the modified method (see Section 7.2.3.) before examining under a Siemen's Elmiskop I transmission microscope.

7.2.3 Staining technique

A saturated filtered solution of uranyl acetate was

prepared using alcohol (100%) which has been reported by Stempak and Ward (1964) to increase the contrast of the specimens. Grids, sections facing downwards, were floated on 1 ml of this solution for 30 min and then washed successively in 70%, 50% alcohol and lastly in glass distilled water twice.

Uranyl acetate staining was then followed by lead citrate stain (Reynolds, 1963). Grids were floated in this stain for 4 min followed by successive washes in 0.05N sodium hydroxide solution (to remove any lead carbonate that may have formed) and twice in distilled water (Mercer and Birbeck, 1972).

7.2.4 Intravascular perfusion fixation

Guinea-pigs were first anaesthetised by an intra-peritoneal injection of pentobarbitone (250 mg/kg). 100 ml of 0.9% saline solution was then perfused into the left ventricle using a hypodermic needle and a large syringe. As soon as the needle was in place the right atrium was cut with a pair of fine scissors to allow venous blood and the perfusate to escape. When most of the blood had drained out, the saline solution was stopped and the glutaraldehyde (2.5%) fixative was then injected into the left ventricle as before. About 200 - 250 ml of the fixative was used. Finally, the cochleas were dissected out and the fixation was continued by the immersion method as described in Section 7.2.1.

7.2.5 Fixatives

15 fixatives were used in this study (Table 26).

Table 26

Different fixatives that were used in this study

	Fixative	Added substance
1.	2.5% glutaraldehyde Intravascular perfusion	None
2.	2.5% glutaraldehyde	None
3.	2.5% glutaraldehyde	2.25% sucrose to both fixatives and the wash buffer
4.	2.5% glutaraldehyde	2.25% sucrose with 2.5mM $CaCl_2$ to both fixatives and the wash buffer
5.	2.5% glutaraldehyde	2.25% sucrose added only to glutaraldehyde fixative and the wash buffer
6.	2.5% glutaraldehyde	2.25% sucrose added only to wash buffer
7.	2.5% glutaraldehyde	4.5% sucrose added to both fixatives and the wash buffer
8.	2.5% glutaraldehyde	4.5% sucrose with 2.5mM $CaCl_2$ to both fixatives and the wash buffer
9.	2.5% glutaraldehyde	4.5% sucrose added only to glutaraldehyde fixative and the wash buffer
10.	2.5% glutaraldehyde	4.5% sucrose added only to wash buffer
11.	2.5% glutaraldehyde	11.06% sucrose added to both fixatives and the wash buffer
12.	2.5% glutaraldehyde	11.06% sucrose with 2.5 mM $CaCl_2$ to both fixatives and the wash buffer
13.	3.0% glutaraldehyde	4.5% sucrose added only to the wash buffer
14.	4.0% glutaraldehyde	4.5% sucrose added only to the wash buffer
15.	5.0% glutaraldehyde	4.5% sucrose added only to the wash buffer

Fresh glutaraldehyde solution was prepared and used in each case.

7.3 Results

Intravascular perfusion

Although the lateral membranes and the sensory hairs appeared normal under the transmission microscope, there was extensive mitochondrial disintegration in both the sensory and the supporting cells (Fig. 29).

2.5% glutaraldehyde

The mitochondria were swollen and in some cases ruptured. Occasional bulging of the lateral membranes was observed. This was considered to be due to the hypotonicity (osmotic effect) of the fixative and the wash solutions. The osmolarity was thus altered by adding sucrose in various amounts to the solutions.

2.5% glutaraldehyde and 2.25% sucrose

Shrinkage of the sensory cells together with mitochondrial swelling and rupture were observed. The lateral membranes did not show any bulging. However, at the apex, the lateral membranes of the outer hair cells were greatly separated. The cytoplasm looked 'empty' and contained a lot of vacuoles. Apart from the nucleus and the mitochondria, the other cellular organelles were unrecognizable. The nucleus appeared to be normal except for the signs of mild oedema (Fig. 30). In all cases both the sensory and the supporting cells were affected.

2.5% glutaraldehyde with 4.5% sucrose

Severe shrinkage of the outer hair cells had occurred with bulging of the lateral membranes although the sensory hairs appeared normal. In places reduced cytoplasmic density was also observed. However, the mitochondria, nuclei and the other cytoplasmic organelles looked normal. The supporting cells and the inner hair cells were unaffected.

Fig. 29 Disintegration of mitochondria (M) observed in an outer hair cell after fixation by vascular perfusion (x5600).

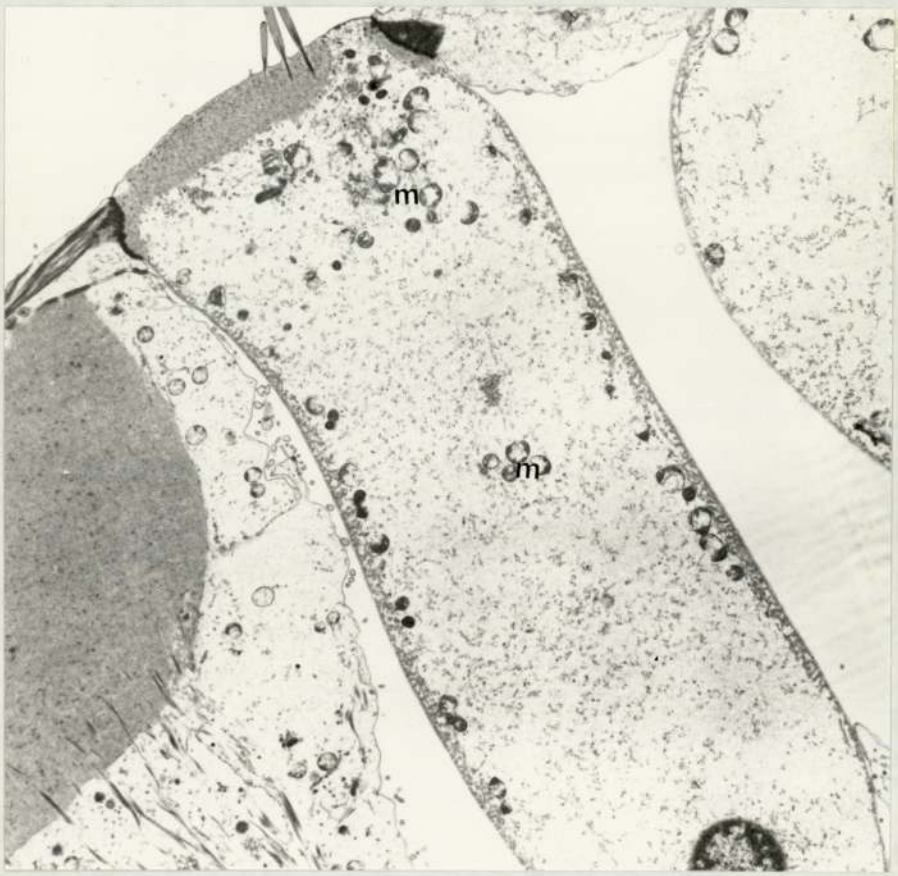
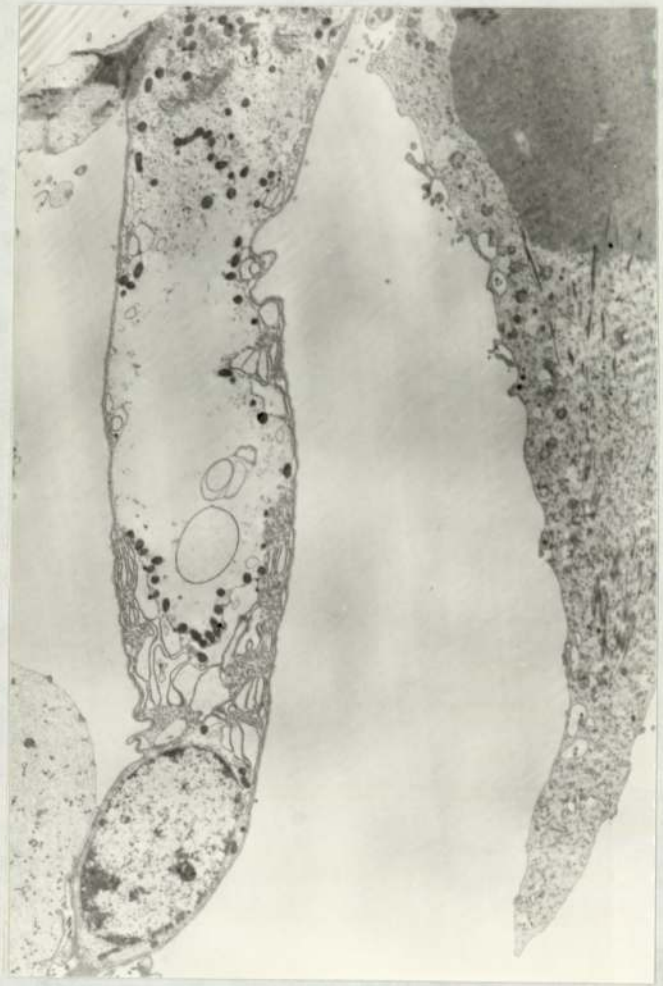


Fig. 30 2.5% glutaraldehyde and 2.5% sucrose.

Outer hair cell from the apical turn showing the separation of the lateral membranes. The cytoplasm looks 'empty' with some vacuoles (x4375).



2.5% glutaraldehyde with 11.06% sucrose

A severe distortion of the sensory cell shape as well as formation of the vacuoles had occurred. In some cases the outer hair cell cytoplasm had split up forming great 'empty' areas (Fig. 31). Extensive bulging of the lateral membranes was observed. However, the mitochondria and the nuclei looked normal.

In the case of the inner hair cells and the supporting cells, extensive vacuolation was present but the actual cell shape was not deformed like the outer hair cells.

3% glutaraldehyde with 4.5% sucrose in wash buffer

Although the outer hair cells looked normal, there were some bulging of the lateral membranes together with some mitochondrial disintegration.

The inner hair cells and the supporting cells were unaffected.

4% glutaraldehyde with 4.5% sucrose in wash buffer

The preservation of all the cochlear cells were excellent and in this study it was regarded as the best fixative for the organ of Corti (Figs. 32 and 33).

5% glutaraldehyde with 4.5% sucrose in wash buffer

The outer hair cells were shrunken with some clearing of the cytoplasm. Some bulging of the lateral membranes was observed. On the whole, the cellular organelles were unaffected (Fig. 34). The inner hair cells and the supporting cells appeared normal.

Fig. 31 Combination of 2.5% glutaraldehyde and 11.06% sucrose. The hair cell cytoplasm is split-up by the formation of great 'empty' areas (x4200).

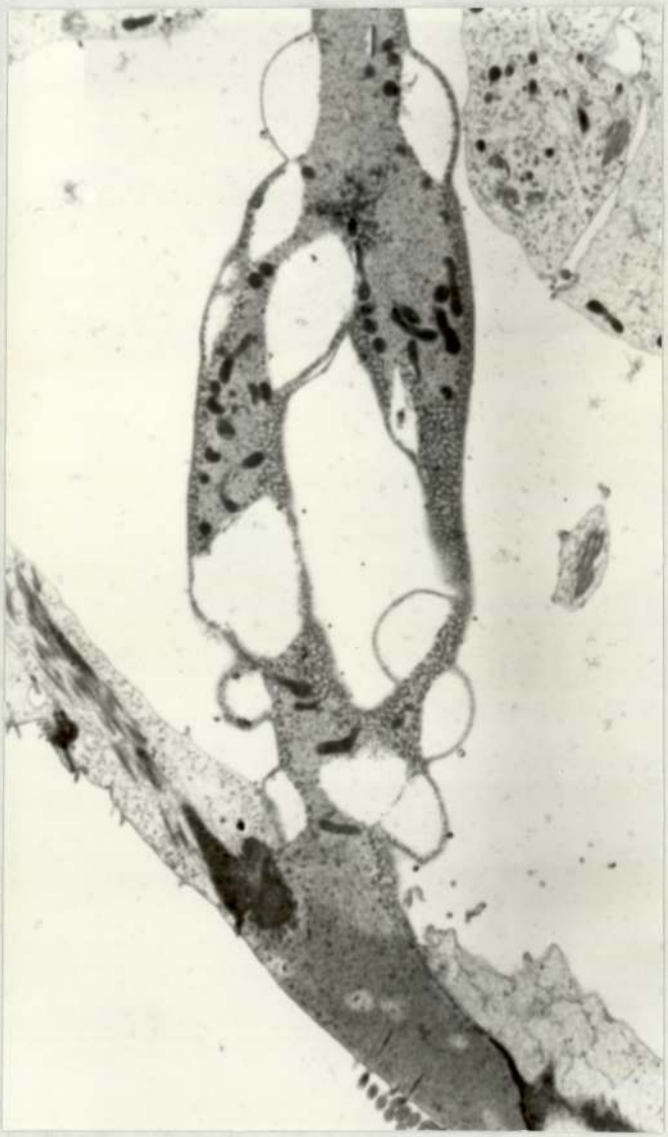


Fig. 32 4% glutaraldehyde with 4.5% sucrose in wash buffer. Upper part of an outer hair cell showing good preservation of the ultrastructure (x4200).

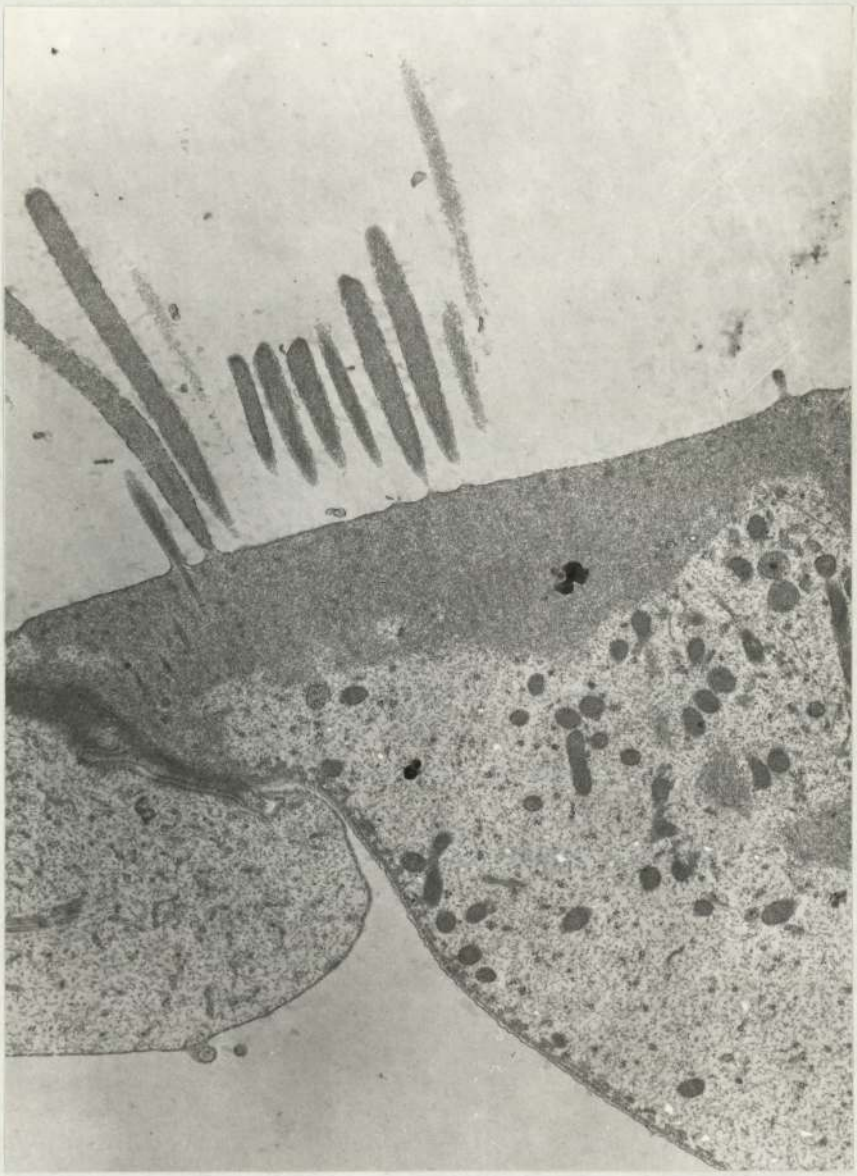


Fig. 33 4% glutaraldehyde with 4.5% sucrose in wash buffer. Inner hair cell showing good preservation of the mitochondria (x5250).

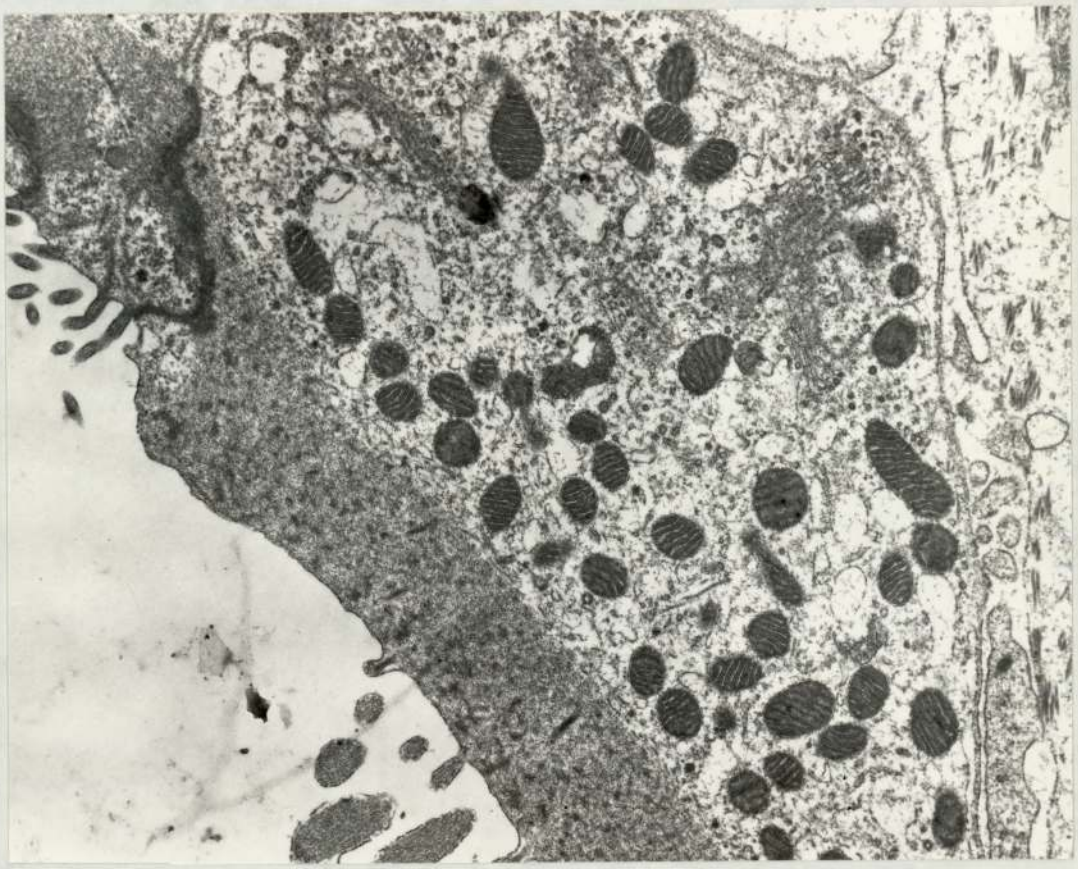
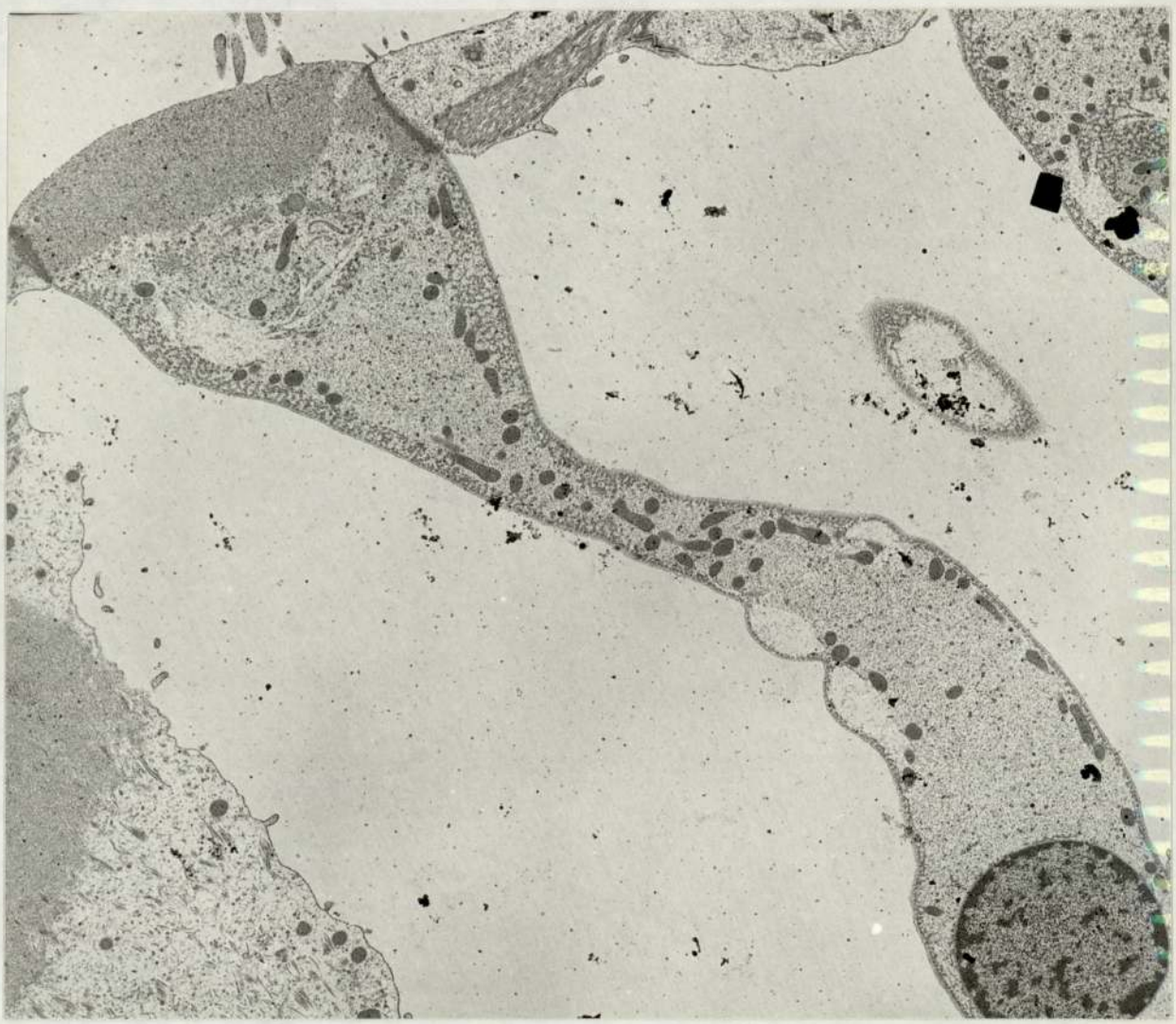


Fig. 34 5% glutaraldehyde with 4.5% sucrose in the wash buffer. The outer hair cell ultrastructure was well preserved except for the appearance of bulging of lateral membranes (x4800).



7.4 Discussion and Conclusions

Analysis of the ultrastructural appearance of the tissues requires an evaluation of how normal cells are influenced by different fixatives before one can apply it to drug-damaged specimens.

One main requirement for proper fixation is the application of the fixative while the cellular metabolism is still normal. For that purpose intravascular perfusion techniques have often been used in recent years with excellent results for tissues such as the kidney (Maunsbach, 1966). In this study, intravascular perfusion was found to be unsuccessful. This could be explained by the fact that the organ of Corti is located sufficiently far from the blood capillaries so that post-mortem changes may have occurred as a result of alterations in cell metabolism before proper concentration of the fixative was attained. Also the fluids surrounding the sensory cells may have diluted the fixative giving rise to these changes (Iurato, 1976).

Fixation by local perfusion followed by immersion was used for the rest of the study. The metabolism of the hair cells of the organ of Corti is dependent mainly on the cortilymph and also on the endolymph to some extent (Lawrence, 1966). Lawrence (1966) demonstrated by selective surgical occlusion of the blood supply to the stria vascularis, that the structure of the organ of Corti remained intact when the stria vascularis had degenerated. However, occlusion of the modiolar vessels going to the capillary loops beneath the basilar membrane resulted in loss of hair cells in the presence of a histologically normal stria vascularis and spiral prominence, indicating that modiolar vessels were the source of nutrients for the organ

of Corti. Anoxia following decapitation of the animal would thus not immediately be mediated to the cochlear sensory cells because of the reserves in the cortilymph and the perilymph. Wersäll et al. (1965) have demonstrated that no ultrastructural changes occurred within 5 minutes following decapitation. The ultrastructural changes observed in this study in some specimens, therefore, were due to poor fixation and not to post-mortem degeneration.

Calcium chloride added to the fixatives has been reported to improve the preservation of mitochondria (Busson-Mabillot, 1971). However, in this study it was found to make no difference.

Of all the fixatives used in the present investigation, the cochlear neuroepithelium was found to be best preserved by a 4% solution of glutaraldehyde as primary fixative followed by post-fixation with 1% osmium tetroxide solution. The wash buffer contained 4.5% sucrose.

The other fixatives gave unacceptable results. On the whole the outer hair cells were more susceptible than the inner hair cells and the supporting cells to ultrastructural changes caused by poor fixation.

SECTION 8

PRELIMINARY STUDIES ON THE POSSIBLE RELATIONSHIP BETWEEN RIBOSTAMYCIN PHARMACOKINETICS AND ITS TOXICITY

8.1 Introduction

8.1.1 Nephrotoxicity

Nephrotoxicity is sometimes regarded as less serious than ototoxicity. Rosenthal (1975) suggested that transient alterations of renal physiology is an inevitable consequence of aminoglycoside treatment and that, if the drug is withdrawn after the effect is recognized, the condition is reversible.

The pharmacological properties of absorption, distribution and excretion of aminoglycosides are almost similar. The drugs are rapidly absorbed following intramuscular injection with the peak serum concentrations occurring 30 - 90 minutes after injection (Riff and Moreschi, 1977). In persons with normal renal function, the half-life of these drugs is approximately two hours (Kunin, 1966; Cabana and Taggart, 1973) and 40% of the drug is excreted in the urine within 24 h (Regamey, Gordon and Kirby, 1973; Kirby, Clarke, Libke and Regamey, 1976; Wood and Farrell, 1976; Jackson, 1977) with a large proportion being excreted during the first 4 h following injection.

Gentamicin, like the other aminoglycosides, is excreted predominantly by glomerular filtration (Riff and Jackson, 1971) and clearance is the same as inulin (Gyselynck, Forrey and Cutler, 1971). A minor role for tubular secretion has been suggested (Gyselynck, Forrey and Cutler, 1971). The drug, like other aminoglycosides, is bound to renal tissue (Luft and Kleit, 1974). During the first 24 h after a single dose, 40%

of the administered dose is excreted in the urine, whereas on subsequent days 85% or more is excreted (Riff and Jackson, 1971). This suggests an initial retention within the body that is relatively independent of renal function. Earlier data by some workers have indicated that approximately 30% of gentamicin in plasma is protein bound (Bulger, Sidell and Kirby, 1963; Riff and Jackson, 1971). Gentamicin protein binding was studied by four different methods. These were gel-filtration, continuous ultrafiltration, frontal analysis and electrophoresis. Results obtained by each of these methods were similar and showed that between 24 to 31% of gentamicin was bound to serum proteins (Section 6, Page 147). Most of the gentamicin was associated with the albumin fraction of the serum.

The incomplete recovery of doses of gentamicin in urine 24 h after a single dose and measurable concentrations of the drug being detected using sensitive assays in the urine of patients with normal function for 20 days or more after the final dose (Kahlmeter and Kamme, 1975) indicates that gentamicin persists in serum and tissues for long periods. Gentamicin, along with other aminoglycosides, has a marked affinity for renal cortical tissue, accumulating to concentrations which are 10 to 50 times those in serum (Kunin, 1960; Rodriguez, Stewart and Bodey, 1970; Edwards, Smith, Baughman, Rogers and Lietman, 1976).

Shentag and Jusko (1977) studied 47 adult patients receiving gentamicin for an average of 10 days. Most of the patients received the drug intravenously, in doses ranging from 0.4 to 7.0 mg/kg/day. Within this range, lower doses were given to patients with renal impairment. Creatinine clearances

were stable and ranged from 8 to 130 ml/min. An average of 24 serum concentrations in each patient were obtained at peak, mid-point and trough times during therapy and on the 28th day after the last dose. Urine concentrations and, in six cases post-mortem tissue concentrations were also obtained. 24 h urinary recovery studies confirmed the incomplete recovery of the daily dosage of gentamicin which had been observed by Riff and Jackson (1971). Total recovery of the drug increased gradually as therapy progressed. Peak and trough concentrations rose gradually in most patients throughout treatment and declined in two phases after the last dose was given. In all 47 patients, the first phase was similar to the apparent decline in concentrations during each dosing interval and seemed to be largely determined by the patients renal function. However, the second phase proceeded more slowly, declining with an average half-life of 112 h (range 27 - 693 h). Post-mortem tissue analysis revealed kidney cortex levels at least 100 times those in serum, with the kidneys alone accounting for 40% of the total amount of gentamicin in the body. The antibiotic remains detectable in serum and urine for long periods because of the slow release from the tissue binding sites which act as reservoirs.

Any drug with a terminal half-life of 112 h is certain to accumulate in the body if given every 8 to 12 h as with gentamicin. This accumulation has been reported to begin with the first dose and continues throughout therapy. Gradual accumulation of this antibiotic may provide an insight into the mechanisms for its nephrotoxicity. It also explains the prolonged detection of gentamicin in various body fluids.

The accumulation of gentamicin by renal tissue may contribute to the reported inability to account for all of the initial dose of gentamicin in urine (Riff and Jackson, 1971), despite the belief (based on the similarity of the renal clearance of gentamicin to that of inulin) that renal elimination by glomerular filtration is the route of excretion of these aminoglycosides (Gyselynck, Forrey and Cutler, 1971). Subsequent doses are apparently easily accounted for, suggesting that the renal parenchyma may rapidly become saturated with the drug. Little is known about the kinetics of this accumulation in human kidneys. It is not known whether repeated doses lead to ever-increasing kidney levels or whether a plateau is reached, nor do we know whether the schedule of administration (e.g. continuous infusion versus intermittent dosing) affects the kidney tissue concentrations achieved in humans. In rats, Luft and his associates (1975) found that renal levels of gentamicin plateaued after 5 days of a 15 day treatment plan. Streptomycin, which is relatively free of nephrotoxicity, has been shown to display a much weaker affinity for renal tissue than do other aminoglycosides (Luft and Kleit, 1974).

Aminoglycosides damage the proximal tubular cells of the kidney, sparing the glomeruli. With moderate doses there is cloudy swelling of tubules, and at higher doses acute tubular necrosis has been produced in animals (Waitz, Moss and Weinstein, 1971). These effects are especially prominent in the proximal convoluted tubules (Harrison, Silverblatt and Turck, 1975) and lysosomal myeloid bodies have been noted in electron microscopical studies in rats (Kosek, Mazze and Cousins, 1974). Myelin figures in the lysosomes of renal

epithelial cells were explained by them to be probably due to phagocytosed residues of damaged cellular organelles. Gentamicin nephrotoxicity has been found to be dose related in animals and acidosis potentiates the renal damage (Hsu, Kurtz, Easterling and Weller, 1974).

The prevalance of gentamicin nephrotoxicity is between 2 to 10% (Hewitt, 1974). Clinical investigations by Hewitt have shown a decrease in probable gentamicin induced nephrotoxicity from 7.7% during the period 1965 to 1966 to 2.9% during 1970 to 1973. He explained that this decrease in frequency of nephrotoxicity may be related to the recognition of patients at high risk, careful monitoring of serum creatinine, performance of serum assays for gentamicin and subsequent adjustment of dosage for gentamicin when it is indicated.

Gentamicin toxicity is manifested by proteinuria and cylindruria (presence of renal cast in the urine) with a rising blood urea nitrogen and creatinine (Wilfert, Burke and Bloomer, 1971; Kleinknecht, Ganeval and Droz, 1973). In general, renal damage is usually reversible if the aminoglycoside is discontinued at first signs of renal dysfunction (Kunin, 1966; Kovnat, Labovitz and Levison, 1973) such as rising blood urea nitrogen, serum creatinine or the presence of protein or tubular cells in the urine (Guisti, 1973). One of the earliest signs of nephrotoxicity is the finding of lysosomal hydrolase enzymes in the urine (Guisti, 1973; Patel, Luft, Yum, Patel, Zeman and Kleit, 1975).

8.1.2 Ototoxicity

Ototoxicity of aminoglycosides has been extensively studied both from the clinical and experimental point of view.

The specific toxicity of these antibiotics for the inner ear has constituted an important clinical problem and the development of new aminoglycosides within related groups has made it necessary to make it a routine to study possible ototoxic effects. Although a few authors have found vascular and neural changes, the consensus of these reports points to the sensory cells of the organ of Corti as the site of primary damage; however, precise information on the exact location of damaged hair cells as regards the respective coil and row of hair cells is generally lacking in these reports.

The ototoxic reactions of several of these antibiotics have been studied in experimental animals with physiological, histological and electron microscopical methods (Duvall and Wersäll, 1964; Kohonen, 1965; Engström and Kohonen, 1965; Wersäll, Björkroth, Flock and Lundquist, 1973; Theopold, 1977) it has become clear that the outer hair cells are, in general, more vulnerable to aminoglycosides than the inner hair cells. However, the functional differences between these two groups of hair cells are not well defined at the present time.

Histopathological studies pertaining to the ototoxicity of kanamycin in guinea-pigs (Hawkins and Engström, 1964; Kohonen, 1965) have revealed a relatively regular pattern and sequence of damage to the cochlear sensory cells by this ototoxic antibiotic. The sequence of sensory cell degeneration was such that a complete destruction of the outer hair cells was first observed in the basal turn. Degeneration then progressed upwards in the cochlea affecting the outer hair cells of the two inner rows in the second coil and of the innermost row in the two apical coils, but at this stage leaving

undamaged the two outer rows in the upper coils of the cochlea. The degeneration of inner hair cells also followed a definite pattern which was found to begin at the apex and progressed towards the base.

Possible explanations of this differential vulnerability of outer hair cells in the different rows and coils of the cochlea have been discussed very little in the literature. It has been suggested by Beck and Krahl (1962) that the slower blood flow in the basal part of the cochlea would result in locally higher concentration of the circulating drugs, and, thus, the cells there might be damaged earlier. However, the clear-cut systematic difference between cells adjacent to each other, but belonging to different rows, can scarcely be explained in this way.

The characteristic behaviourial effect of the ototoxic antibiotics is the rise in threshold for the higher frequency pure-tones. Thus, there is a good agreement between morphological damage to the basal outer hair cells and functional disruption of the frequencies they serve.

Kohonen (1965) studying neomycin, kanamycin and framycetin ototoxicity in guinea-pigs reported that the most characteristic ototoxic effect seen was the collapse of the sensory cells. Discussing such damage, he described the swelling of the hair cell nuclei and their breakdown. Other workers (Wersäll and Lundquist, 1968) have presented detailed descriptions of hair cell damage listing degeneration of mitochondria, fusion of the sensory hairs, changes in cell membrane, and bulging of the cell towards the endolymph under the influence of streptomycin. Wersäll and Lundquist (1968) divided

kanamycin-induced hair cell damage into stages, listing nuclear and ribosomal changes as early reversible and mitochondrial and cell-membrane changes in the late irreversible stage. Supporting cells, ganglion cells and efferent endings were all reported to be less susceptible than the hair cells to damage by antibiotic ototoxins. The initial changes in the nuclei and in the ribosomes of the cells indicate an inhibition of RNA synthesis in the sensory cells.

That hair cell degeneration is secondary to action elsewhere in the cochlea has been suggested by Hawkins (1970). Among the suggested primary sites are the stria vascularis especially the mitochondria of the stria vascularis, the pericapillary elements of the outer sulcus with damage extending throughout the spiral ligament, spiral limbus and Reissner's membrane (Hawkins, 1970). The stria vascularis, elements of the spiral ligament and the Reissner's membrane are all involved in secretion or filtration, and a disruption of this function might easily result in severe changes in the environment of the hair cells. Such a change in hair cell environment could account for the diminution in cochlear microphonics as well as the observed morphological changes. There are some studies on the accumulation of aminoglycosides in the ear (Voldrick, 1965; Stupp, Rauch, Sous, Brun and Lagler, 1967; Stupp, Küpper, Lagler, Sous and Quante, 1973; Federspil, Schatzle and Tissler, 1976; Brummett, Fox, Bendrick and Himes, 1978). They all demonstrated that aminoglycosides penetrate the inner ear via the blood circulation and then accumulate in endolymph and perilymph. They are eliminated slowly. The high concentration and prolonged presence in the inner ear

fluids may account for the specific ototoxic effect of these substances. Stupp et al. (1973) applied an intramuscular injection of each of the aminoglycosides neomycin, dihydrostreptomycin, kanamycin and streptomycin to the guinea-pigs. Their concentration in the serum, endolymph and perilymph of the inner ear was determined over a period of 25 h. High antibiotic concentration levels were found in the perilymph, with concentrations in the endolymph almost as high. Besides the high concentrations of the antibiotics in the inner ear, the extremely long duration in the inner ear was striking. For a short time the concentrations in the serum were very high, the half-life of the antibiotics here was only 80 min. In the inner ear, however, the half-life measured was approximately 15 hr which was 10 times longer. 25 h after the application of the antibiotics, they could still be detected in the perilymph. This observation could also be confirmed by Voldrich (1965).

Ototoxicity was explained by Stupp et al. (1973) to be due to high concentrations and the persistance of the antibiotics in the inner ear. The antibiotics of the aminoglycoside group have varying strong ototoxic characteristics. Accordingly, they found that the less toxic antibiotics showed a substantially lower concentration in the inner ear. The highest concentration in the inner ear was found with neomycin which proved to be the most toxic thus indicating that drug accumulation in the perilymph is apparently related to the ototoxicity of these drugs (Brummett et al., 1978).

Under normal conditions, the inner ear maintains voltage differences between different compartments. These voltage

differences depend on the maintenance of the distinctive differences in composition between endolymph and perilymph. Integrity of Reisnner's membrane is obligatory for maintaining the endolymph-perilymph composition. The inhibitory influence of streptomycin on guinea-pig cochlear microphonics (CM) was reversed by potassium (Wersäll and Lundquist, 1968). This reversal of streptomycin-induced reduction in CM by washing the endolymph compartment with high potassium Ringer's solution suggests the possibility that these antibiotics may be acting to inhibit the cochlear $Na^+ - K^+$ ATPase. The demonstration by Iinuma (1966) that kanamycin intoxication in guinea-pigs produced a reduction in ATPase activity of the stria vascularis and spiral ligament provides more circumstantial evidence linking ototoxicity and membrane ATPase. Therefore, the correlation between the ototoxicity of aminoglycosides and thier ability to inhibit the membrane ATPase, although not conclusively demonstrated, remains tantalizing.

Another captivating suggestion regarding the mechanisms of ototoxicity stems from the observation that aminoglycoside antibiotics contain an aminosugar in their structure. Certain aminosugars are known to reduce the uptake of metabolically important molecules such as glucose (Balazs and Jacobson, 1966). Although a mild reduction in glucose uptake in organs such as liver may not be of importance to the economy of these organs because they operate with large metabolic reserves, a similar reduction in uptake in the cochlea, reducing glucose availability to the hair cells may be critical. The metabolic reserve of the hair cells may be too small to survive reduction in supply of glucose. Such a small metabolic reserve would

then 'select' the hair cells as the structures most susceptible to the ototoxic antibiotics.

Chemically, the aminoglycosides have strongly basic groups. The ability to cause nephrotoxicity is not fully correlated with ototoxicity but it is regularly seen with antibiotics containing amino groups. However, it seems reasonable to assume that the cochlea and kidney share certain drug-vulnerable processes. Furthermore, it is now clear that ototoxicity of aminoglycosides is potentiated by diminished renal function, presumably because of the failure to excrete the drug with resultant prolonged exposure of cochlear structures to increased concentrations.

This study was designed to investigate the toxicity of ribostamycin in relation to its kinetics in the perilymph and serum. The aminoglycoside ribostamycin is produced by Streptomyces ribosidificus. The in vitro activity of this drug has been reported to be comparable to or slightly weaker than kanamycin but it causes lower ototoxicity (Harada, 1972) and nephrotoxicity (Kawagishi, Nagamatsu, Nakajima, Doi, Yamada and Yamamoto, 1972).

Harada (1972) observed no evidence of ototoxicity when 400 mg/kg/day of ribostamycin was administered intramuscularly for four weeks. Audiological, electrophysiological and histological techniques were applied for the investigation of ototoxicity. On the other hand, with kanamycin administration, 70% of the animals demonstrated toxic effects on the ear. When 200 mg/kg of ribostamycin was administered intramuscularly for 22 days, no toxic effects on the kidney were detected using histopathological methods (Kawagishi et al., 1972).

Originally, the idea was to perform an acute and chronic study using both gentamicin and ribostamycin at two dose levels (200 and 400 mg/kg). Perilymph and serum samples would be collected at eight time intervals. Three guinea-pigs would be used at each time point. It was calculated that 96 guinea-pigs would be required for the whole study. The toxicity of ribostamycin was to be compared with gentamicin because most of the previous studies on comparative ototoxicity of aminoglycosides have used gentamicin as standard against which all other aminoglycosides were compared with. Also, gentamicin is the only aminoglycoside which is currently in use worldwide. Economically and physically it was impossible to do this. The revised protocol was then drawn out and was used to study the kinetics of ribostamycin alone in both the perilymph and the serum of the guinea-pigs. The idea was to relate the toxicity of the drug to its kinetics.

Preyer reflex threshold was used to monitor auditory function. Preyer's reflex is the twitch of the animal's pinna in response to a loud sound and was used as a measure of hearing. Preyer reflex threshold is normally at least 70dB above the absolute threshold of hearing. This method will therefore detect only a reduced sensitivity to intense sounds which will normally occur only when a hearing impairment has progressed to extreme limits. However, the advantage of this response is that it permits serial study of an animal's hearing before, during and after administration of the drug. The test is simple thus making it possible to examine large numbers of animals daily. The presence or absence of the Preyer reflex at each sound intensity is

assessed by visual observation.

Preyer reflex threshold method of assessment of auditory function has been applied successfully by Carter (1979). He described it as a reliable and a valid method of assessment of the relative cochleotoxic potentials of aminoglycosides.

The present study was performed in two phases:
The acute and the chronic phases.

The objectives of the acute study were:

1. To determine the pharmacokinetic profile of ribostamycin in both serum and perilymph after a single (400 mg/kg) subcutaneous injection.
2. To measure the serum creatinine concentrations as baseline data for the chronic study.

The objectives of the chronic study were:

1. To determine whether 400 mg/kg of ribostamycin administered subcutaneously daily for 14 days was ototoxic and/or nephrotoxic as indicated by rises in Preyer reflex threshold or serum creatinine concentration respectively. Post-mortem histopathological investigation of the cochlear and renal tissues was also anticipated.
2. To determine whether there was a relationship between the pharmacokinetics of ribostamycin and any observed toxicity by:

a. measuring the pharmacokinetic profile of ribostamycin in serum and perilymph immediately following the last of the 14 daily injections.
b. comparing these profiles with those found after single dose administration to determine whether any accumulation had occurred in either serum or perilymph.
c. measuring the serum and perilymph ribostamycin concentrations

at least two weeks after drug administration had been stopped to ascertain whether there was retention of the drug.

8.2 Methods and Materials

A total of 86 healthy male albino guinea-pigs with a normal Preyer reflex weighing around 450 g were used in this investigation. 39 were used in the chronic dosage experiment and 47 were used in the acute experiment where the pharmacokinetics of a single dose were investigated. All drugs were given as daily subcutaneous injections at the dosage of 400 mg ribostamycin base/kg body weight. The control group were given vehicle.

For the acute pharmacokinetic study, each animal received a single injection of 400 mg/kg of ribostamycin. Blood and perilymph samples were obtained at $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 4, 8, 12 and 24 h after the dose. Three out of the 24 guinea-pigs were assigned to each of eight time periods. Animals were anaesthetised with sodium pentobarbitone (10 mg/ml) before samples were taken. Samples of blood were collected by cardiac puncture. The stapes were removed and samples of perilymph were then collected from each cochlea using micropipettes inserted through the oval window membrane. Four animals were used as controls.

For the dose-response study, 3 out of the 12 guinea-pigs were assigned to each of the four dose levels of ribostamycin. The dose levels used were 100, 200, 300 and 400 mg ribostamycin base/kg body weight. 12 h after the injection, the animals were killed and the perilymph samples were collected in exactly the same manner as described above and the amount of ribostamycin present in each of the perilymph samples was assayed by the microbiological assay.

39 animals were used for the chronic pharmacokinetic study. 9 of the 39 guinea-pigs were used as controls and

were given subcutaneous injections of 0.1 ml of 0.9% saline per 100 g of body weight daily for 14 days. Each of the other 30 guinea-pigs received daily subcutaneous injections of 400 mg ribostamycin base/kg body weight for 14 days. The drug doses were weight-adjusted for each animal everyday. Preyer reflex threshold was tested at the time intervals of 1, 2, 3, 5, 7, 9, 10, 12 and 13 days. After the last injection, three ribostamycin and three control animals were kept for an additional two weeks; three ribostamycin injected and three control animals were kept for an additional four weeks for stabilization of any ototoxic effects produced and also to ascertain whether there was any retention of ribostamycin. In the latter group, one control animal died on day 30; this was the only mortality.

3 out of the 24 guinea-pigs were assigned to each of the eight time periods and after the last injection of ribostamycin, samples of blood and perilymph were obtained at the same time intervals as the acute study. Three animals were used as controls.

8.2.1 Drugs

Ribostamycin sulphate solutions were prepared in distilled water in terms of base. Doses contained in the volume of 0.1 ml per 100 g of body weight were administered subcutaneously. The control group received 0.1 ml of 0.9% saline per 100 g body weight.

8.2.2 Assay of ribostamycin

Tha samples of serum and perilymph were assayed by the microbiological assay method using Bacillus subtilis ATCC 6633 as the indicator micro-organism. The bioassay was carried out

by Pfizer Ltd., Central Research, Sandwich, Kent. Serum samples were diluted in normal rat serum and assayed in triplicate against standards which had been similarly prepared. Perilymph samples were diluted 10-fold in phosphate buffer (pH 8) and assayed in a single determination against standards prepared in the same buffer. With 1 µl of sample, the limit of detection by this method was 0.3µg/ml.

8.2.3 Kidney function

Serum creatinine and blood urea nitrogen (BUN) levels were the two parameters used to estimate kidney malfunction.

8.2.4 Kidney pathology

Kidneys were halved and fixed for 4 h in Bouins fixative. They were subsequently stored in 70% alcohol until processed. The material was embedded in 'Paraplast' and sectioned with a microtome. Haemotoxylin and eosin stains were used for staining the sections. Histological studies on the kidneys were carried out by Pfizer Ltd., and were performed blind.

Renal changes were evaluated by the incidence and severity of tubular changes. The histological appearances which acted as marker lesions were tubular dilatation, deposition of material within the tubular lumen, tubular epithelial exfoliation, cytoplasmic degeneration, focal and diffuse necrosis and interstitial nephrotis. The combination of these changes with differing severity gave an indication of renal toxicity and were scored on a 0 - 5 scale.

8.2.5 Auditory function

The apparatus used consisted of a Levell R.C. Oscillator (type T.G.200; Levell Electronics Ltd., Barnet, Hertfordshire, England) which delivered a pure sine-wave signal of adjustable

frequency of a 15 watt amplifier/control unit designed and constructed by Pfizer Central Research Instrument Laboratory, Pfizer Ltd., Sandwich, Kent, England. The sound stimulus was emitted by a Goodmans magnum K2 speaker system in pulses of 0.2 second duration at 1 second intervals.

The threshold sound intensity (dB re. 2×10^{-5} N/m^2 at 15 cm) forPreyer's reflex was measured by positioning each animal, with light manual restraint, with his ears 15 cm from the speaker. The oscillator was set at 2000 Hz, and the volume was increased to elicit Preyer's pinna reflex. The volume at which the pinna reflex was elicited for three consecutive times was recorded. The volume setting at which this occurred was converted to decibels by means of a calibration chart and taken as the threshold sound intensity. This process was repeated at frequencies 4000, 8000, 12000 and 16000 Hz. The guinea-pig's auditory function was measured at intervals of 1, 2, 3, 5, 7, 9, 10, 12 and 13 days. Animals which were kept for an additional two or four weeks were tested every alternate day thereafter. All hearing tests were performed in sound-proof room and were conducted by Dr. E. S. Harpur.

8.2.6 Cochlear pathology

After decapitation, the cochleas were immediately removed, and after perilymph extraction, fixed successively in 1.5% phosphate-buffered glutaraldehyde and osmium tetroxide. They were then transferred into 50% alcohol for 15 min and then to 70% alcohol. In 70% alcohol, cochleas were dissected systematically in half turns using the same procedure as that mentioned in Section 7, Page \87. The specimens were mounted in 50% glycerol and the surface preparations were examined under

the phase contrast microscope (Zeiss). In order to characterize the lesions more precisely, inner and outer hair cells were counted individually and plotted in a form of a cytocochleogram. This is described below fully. Selected specimens were then examined under the scanning electron microscope (SEM) and transmission electron microscope (TEM). In the case of SEM, tissues were dried on a critical point drier and sputter coated with a layer of gold (Anderson, 1951). The samples were then viewed in a Cambridge SEM (S100). Tissues for TEM studies were prepared in the same manner as mentioned in Section 7, Page 187. Studies on cochlear pathology were conducted blind.

8.2.7 The cytocochleogram

By the aid of the surface specimen technique, it was possible to survey large portions of the organ of Corti in normal control as well as in cochleas from drug-injected animals. It was easy to localize individual cells undergoing degeneration and, because of the orderly arrangement, such cells could be easily registered in relation to adjacent normal cells. In this way, cellular loss could exactly be recorded in individual experimental animals. Specimens were taken from corresponding regions in each of the cochleas so that a direct comparison of the extent of damage could be made from one animal to another. Thus, the cytocochleogram represented a schematic graphic representation of the arrangement of sensory cells, where each cell, normal or damaged had its correct place in the pattern. Normal cells were registered as open circles (○) and the missing cells as solid circles (●).

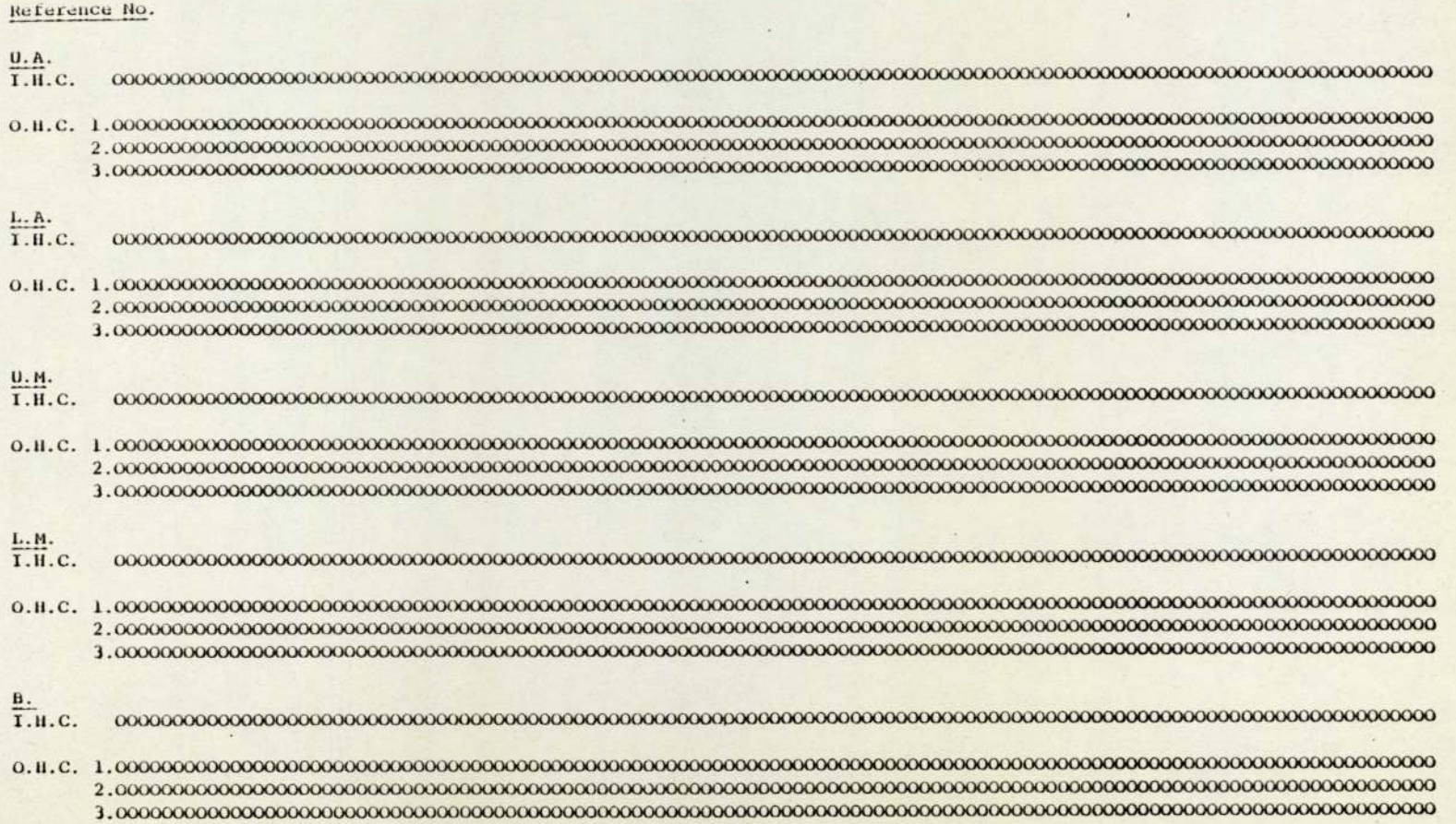
In practice, the recording of the sensory cell damage in the cytocochleograms was made in the following manner: From

each fixed cochlea, a representative segment from each coil was examined. These specimens usually around one-third of the length of the coil, were taken from the lateral part of the cochlea. The origin of these specimens was noted so that each specimen originated from the basal (B), lower middle turn (LM), upper middle turn (UM), lower apex (LA) and lastly the upper apex (UA). The reason for using such terms was to emphasize that each specimen originated not only from a certain coil, but from a certain part of the coil, which represented the same location in every cochlea. This standardization is of crucial importance when results from different animals are compared.

Each specimen containing three rows of outer hair cells and one row of inner hair cells was used. 115 cells were counted in each row which corresponded to roughly 1 mm length of the organ of Corti. Therefore, each region included in the cytocochleogram contained 460 sensory cells exactly plotted in their natural interrelation. Each cytocochleogram included five separate samples and the complete diagram thus included 2,300 sensory cells, which was considered a fair, standard sampling of cellular damage (Fig. 35). The total number of cells in the whole cochlea are considered to be in the region of 15,500. Therefore, the percentage of total cells counted was calculated to be about 15%. The disadvantage of this method of counting damaged and normal cells is that one can never be sure from the presence of a hair cell that it is still able to function normally.

Fig 35. The cytocochleogram as used to register the hair cell loss.

O.H.C. = outer hair cell; I.H.C. = inner hair cell; B = base; L.M. = lower middle turn; U.M. = upper middle turn; L.A. = lower apex; U.A. = upper apex turn.



8.3 Results

Results showed that following a single injection of 400 mg of ribostamycin subcutaneously, there was rapid rise after 30 min to a peak level of 592 µg/ml in the serum of the drug. Its rate of excretion was such that within 24 h after administration, it had been cleared almost completely from the serum (Fig. 36, Table 27). The concentration of the drug in perilymph increased slowly reaching a peak level between 2 to 8 h (Fig. 36, Table 28). There was distinct retention of ribostamycin in the inner ear fluids even after it had been cleared from the serum. This retention was emphasized by comparison of the pharmacokinetics in perilymph with that in serum in which no drug was retained.

The dose-response experiment showed that the concentration of ribostamycin in perilymph was linearly related to the size of the dose (Fig. 37).

The BUN and serum creatinine values at the end of each time period from ¼ - 24 h showed that by the end of 2 h there was no change in BUN and creatinine levels, but the BUN levels from 4 h onwards appeared to be significantly elevated whereas creatinine levels remained unchanged throughout. Even though the ribostamycin had been cleared from the serum over a period of 12 h, there were changes in the BUN levels.

Light microscopic appearances of the kidney tissues showed that alterations were minimal and there was no indication of specific nephrotoxicity. The incidence of single and scattered necrotic cells in the renal tubules increased in the 30 min, 1 h and 2 h groups and there were increases in severity of tubular degeneration within the same

Fig. 36 Comparison of concentrations of ribostamycin in serum and perilymph after a dose of 400 mg/kg. (Mean ± S.D)

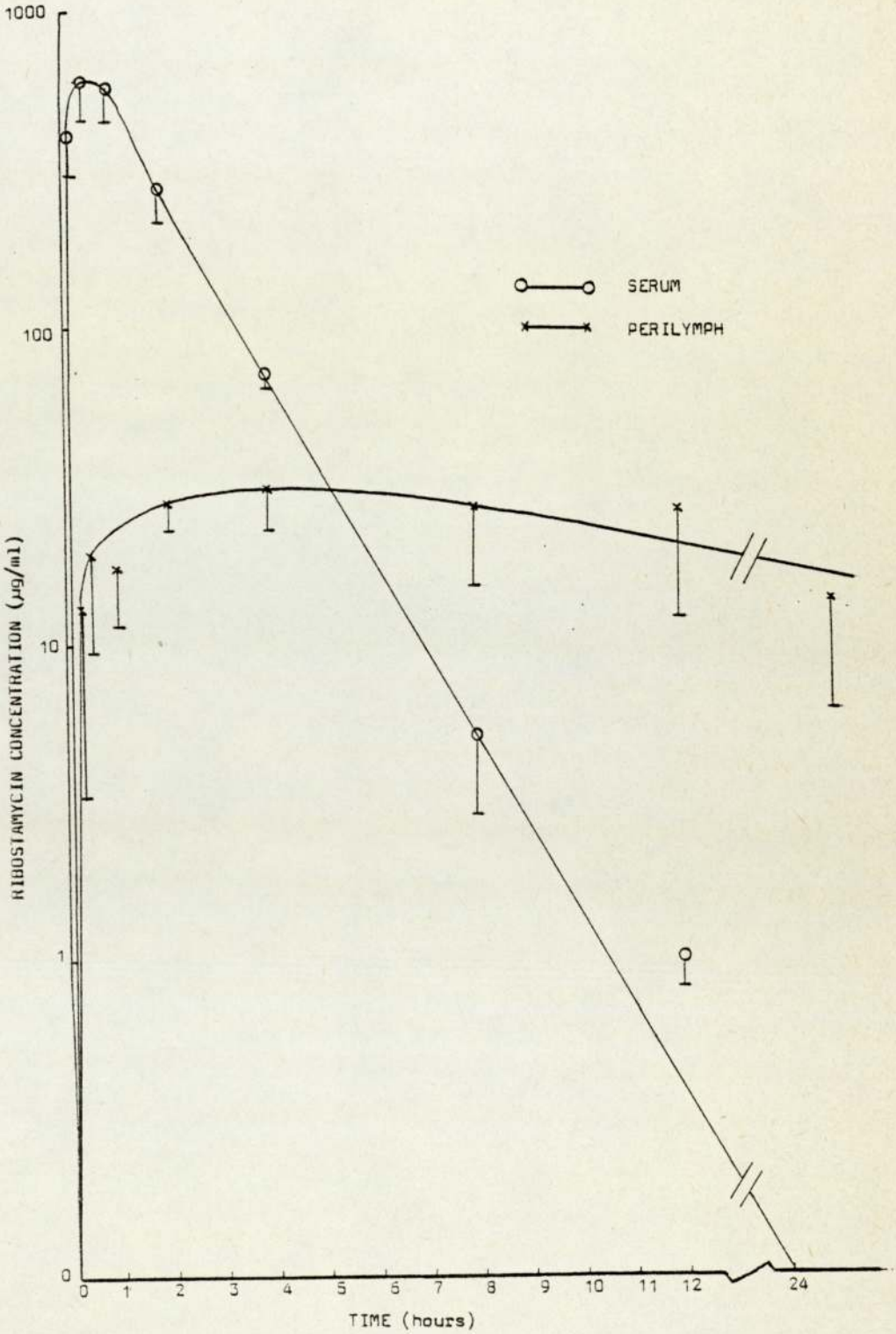


Table 27

The concentration of ribostamycin in the serum after a single subcutaneous injection of 400 mg/kg body weight of the drug

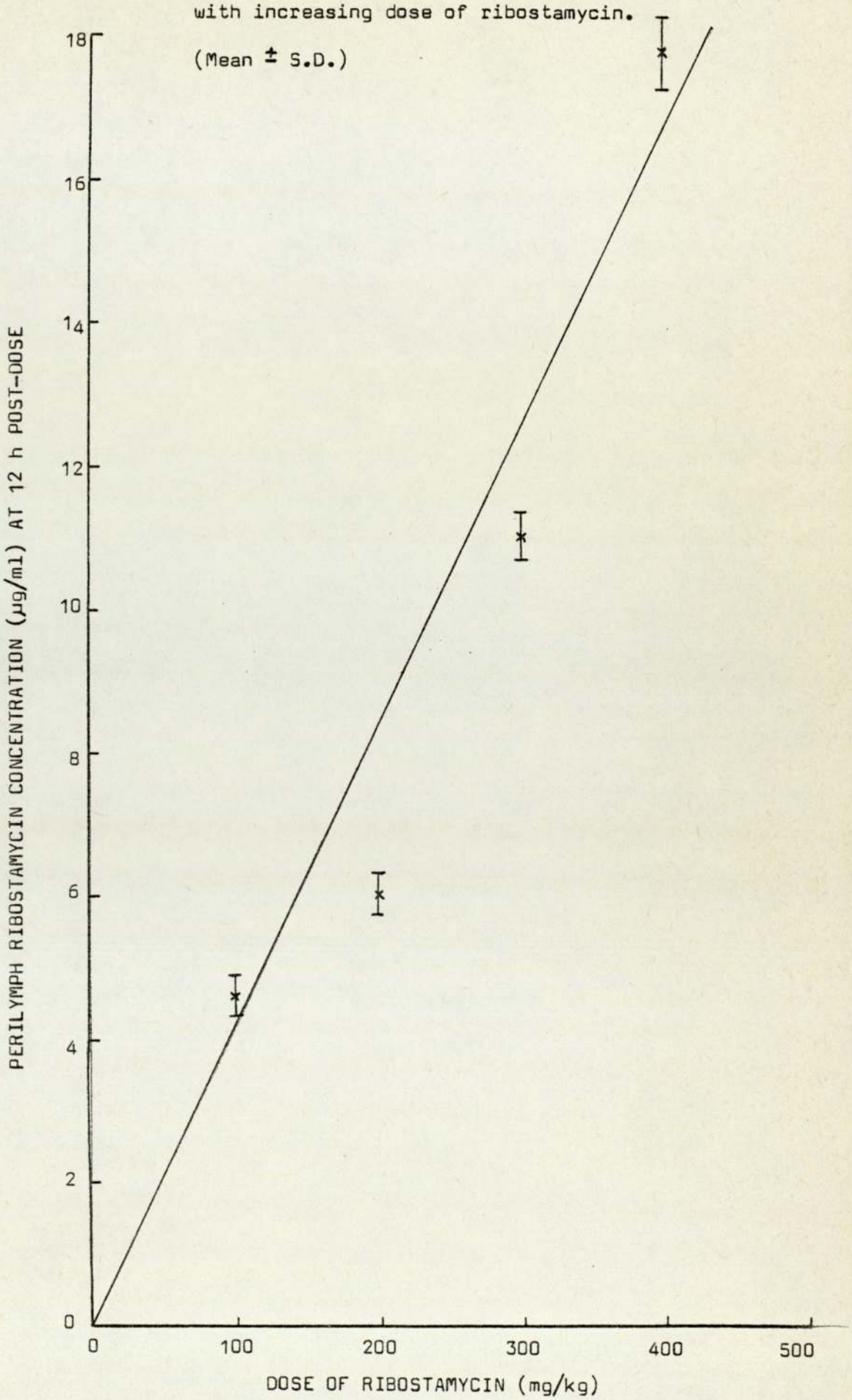
TIME	MEAN (µg/ml)	S.D
15 min	392.0	98.0
30 min	592.0	129.0
1 h	479.0	177.0
2 h	281.0	52.0
4 h	57.0	18.0
8 h	5.0	2.0
12 h	0.8	1.1

Table 28

The concentration of ribostamycin in the perilymph after a single subcutaneous injection of 400 mg/kg body weight of the drug

TIME	MEAN (µg/ml)	S.D
15 min	12.5	9.2
30 min	19.4	10.8
1 h	17.8	6.1
2 h	28.2	4.9
4 h	31.5	9.3
8 h	26.6	12.0
12 h	25.2	14.1
24 h	13.4	7.9

Fig. 37 Graph showing increasing perilymph concentrations with increasing dose of ribostamycin. (Mean ± S.D.)



periods.

Histopathological studies of the cochleas using both the phase contrast and the transmission electron microscopes demonstrated no changes from normal control animals.

Following 400 mg/kg doses fro 14 days, the peak level of ribostamycin was reached rapidly at 15 min. The drug was excreted mainly over the first 4 h with just detectable levels (0.3 µg/ml) at 24 h (Fig. 38, Table 29). The pharmacokinetic profile was similar to that of following a single dose of 400 mg/kg (Fig. 39). Perilymph levels were found to be (Table 30) essentially the same as for a single dose of ribostamycin (Fig. 39). Multiple-dosing, therefore, did not result in the accumulation of ribostamycin in the perilymph.

BUN and creatinine levels did not appear to be elevated at any time interval during the chronic experiment. Histopathological studies on the kidney indicated some focal proximal tubular degeneration and interstitial nephritis in animals killed at 4 and 8 h after the last injection. However, these pathological changes were less severe in animals killed 12 and 24 h after the last injection. Animals killed 2 and 4 weeks after the last injection did not demonstrate any pathological changes.

The threshold sound intensity for Preyer's reflex did not show any changes in hearing during the 14 days of treatment with ribostamycin. Animals which were allowed to survive for 4 weeks showed an increase in threshold at all frequencies compared to the control animals. This change occurred 15 days after the drug had been stopped (Fig. 40). The highest threshold shift which was found to be of the order of 10dB was

Fig. 38 Comparison of the concentrations of ribostamycin in the serum and the perilymph after 14 daily injections of 400 mg/kg of ribostamycin. (Mean ± S.D).

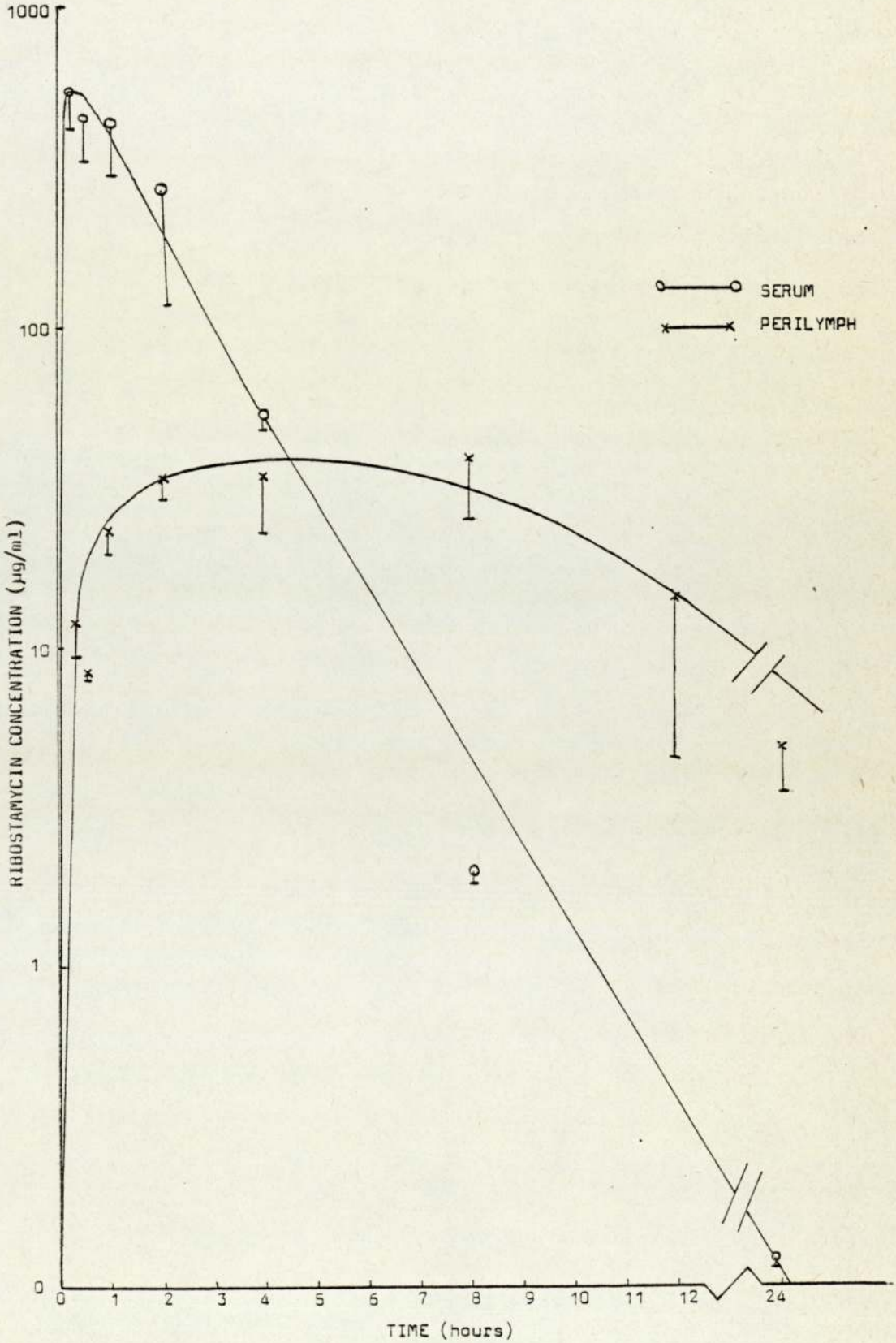


Table 29

The concentration of ribostamycin in the serum after fourteen subcutaneous injections of 400 mg/kg body weight of the drug daily

TIME	MEAN (µg/ml)	S.D
15 min	553.0	92.0
30 min	440.0	106.0
1 h	420.0	121.0
2 h	262.0	148.0
4 h	56.0	30.0
8 h	2.0	2.0
12 h	0.9	0.4
24 h	0.3	0.1

Table 30

The concentration of ribostamycin in the perilymph after fourteen subcutaneous injections of 400 mg/kg body weight of the drug daily

TIME	MEAN (µg/ml)	S.D
15 min	12.1	3.5
30 min	8.1	0.8
1 h	23.8	4.0
2 h	34.4	5.0
4 h	34.1	17.6
8 h	39.4	14.6
12 h	14.8	10.5
24 h	4.9	1.4

Fig. 39 Graph showing the concentration of ribostamycin in serum and perilymph after a single dose (400 mg/kg) and after fourteen doses (400 mg/kg daily) of ribostamycin administered subcutaneously.

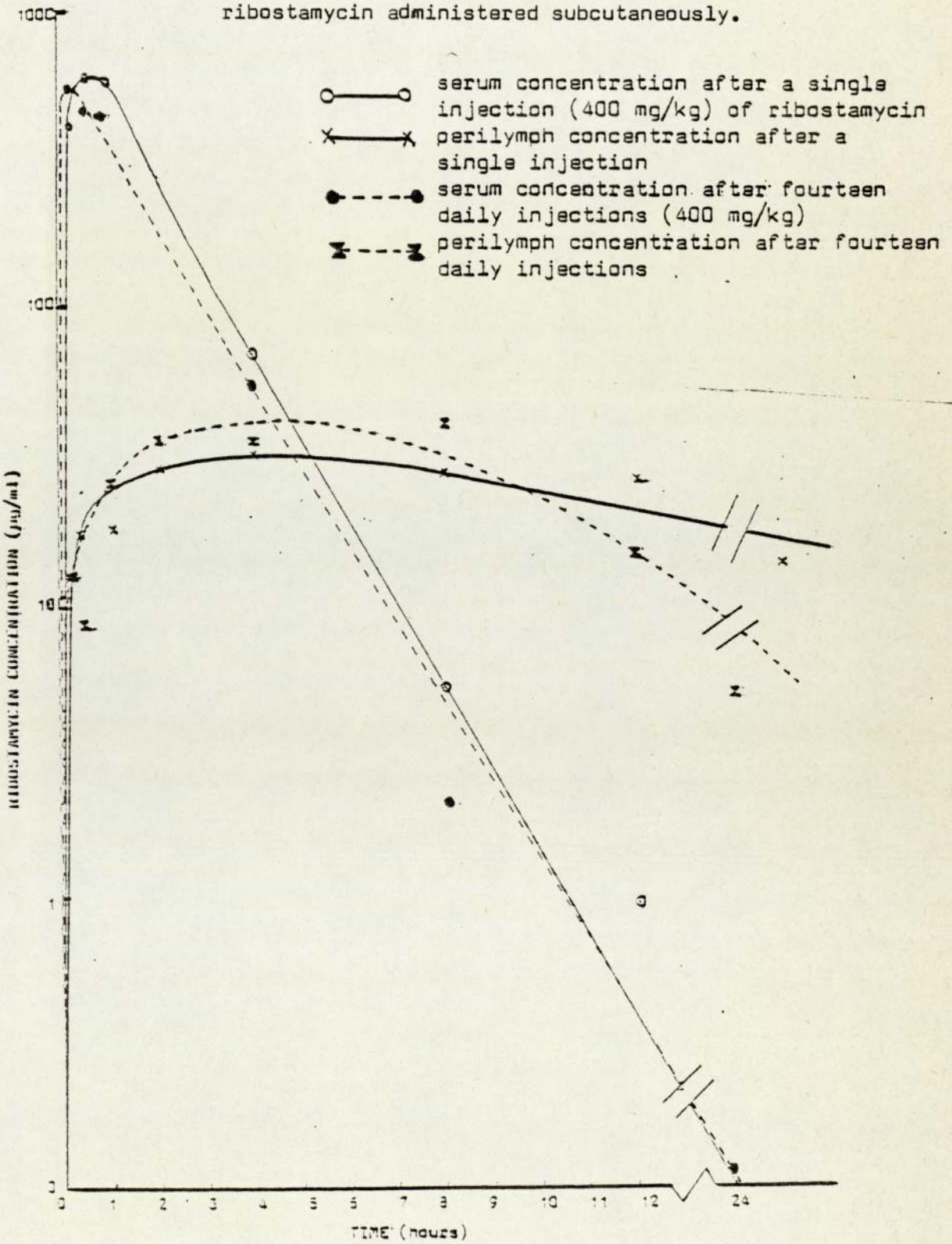
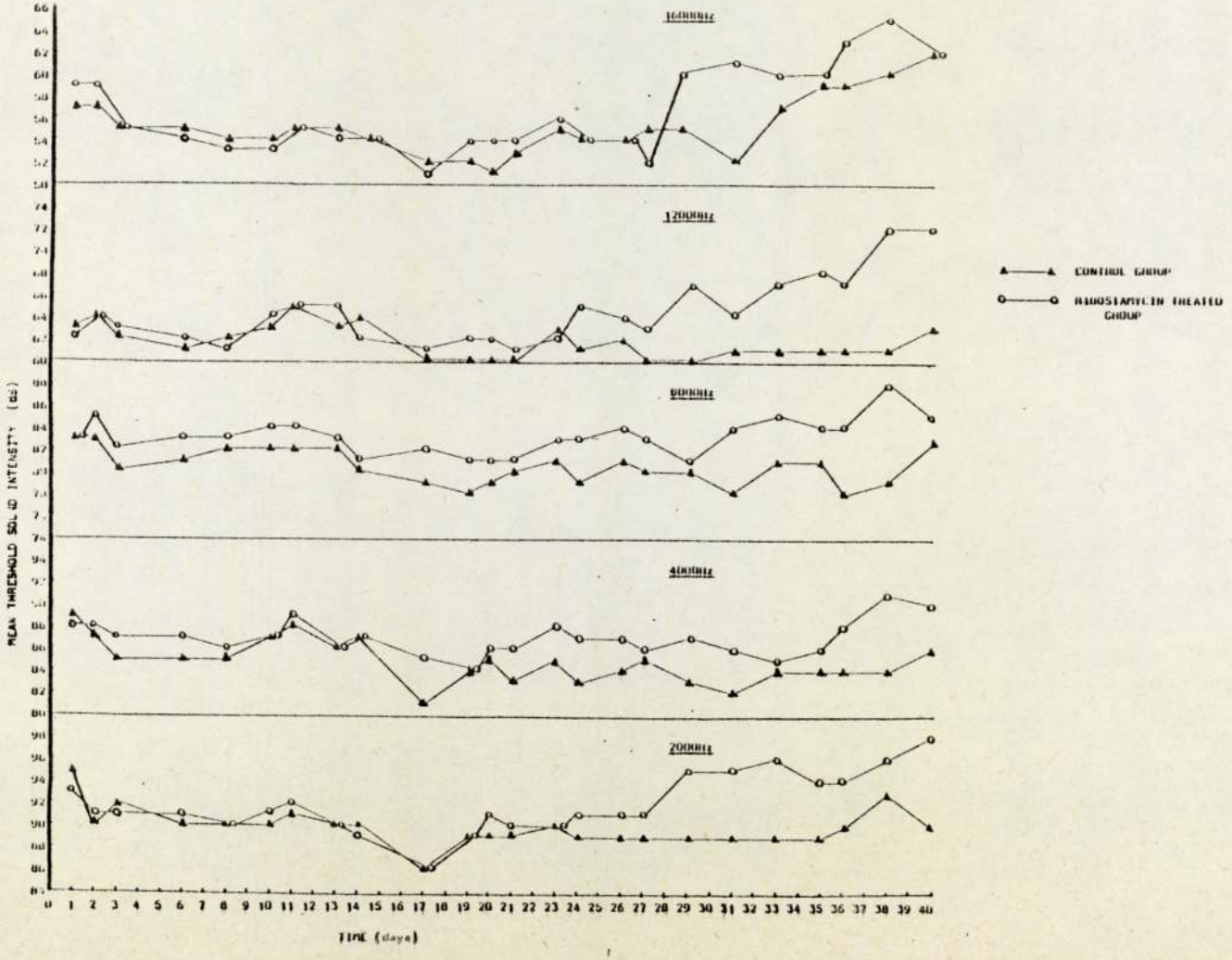


Fig. 40 Effect of ribostamycin on the mean threshold sound intensity for Preyer's reflex at frequencies 2000 to 16000Hz in guinea-pigs. Guinea-pigs had been injected with ribostamycin for 14 days (400 mg/kg body weight daily).



observed at 12000Hz frequency (Fig. 41). The uninjected control group animals did not demonstrate any changes throughout the experiment.

Examination of the surface preparations revealed a scattered loss of single outer hair cells at all coils. Occasionally, there was a single inner hair cell missing (Fig. 42). On the whole there was no definite pattern in the distribution of the missing cells. The highest mean hair cell loss was observed in the animals killed 2 weeks after the last injection had been stopped. This was calculated to be 0.6% compared to 0.2% in the control animals which was found to be non-significant statistically. A cytocochleogram showing the typical hair cell loss observed in this group is shown in Figure 43. Changes in the disarrangement of the pattern of sensory hairs on individual outer hair cells was observed in two of the guinea-pigs in the group which had been allowed to survive for 2 weeks after the last injection had been stopped and in three guinea-pigs which had been allowed to survive for 4 weeks after the last injection had been stopped. In normal specimens the 'W' pattern formed by the sensory hairs always showed a uniform conformation (Figs. 44 and 45) but in these long term survival groups, the 'W' pattern had become irregular lines with hairs often pointing in different directions (Fig. 46). This phenomenon was first observed under the phase contrast microscope and was later seen under SEM.

With the TEM, the major change was also found to be disarrangement of the sensory hairs of the outer hair cells (Fig. 47) in the animals which had been allowed to survive

Fig. 41 Change in mean threshold sound intensity for Preyer's reflex at 12000Hz.

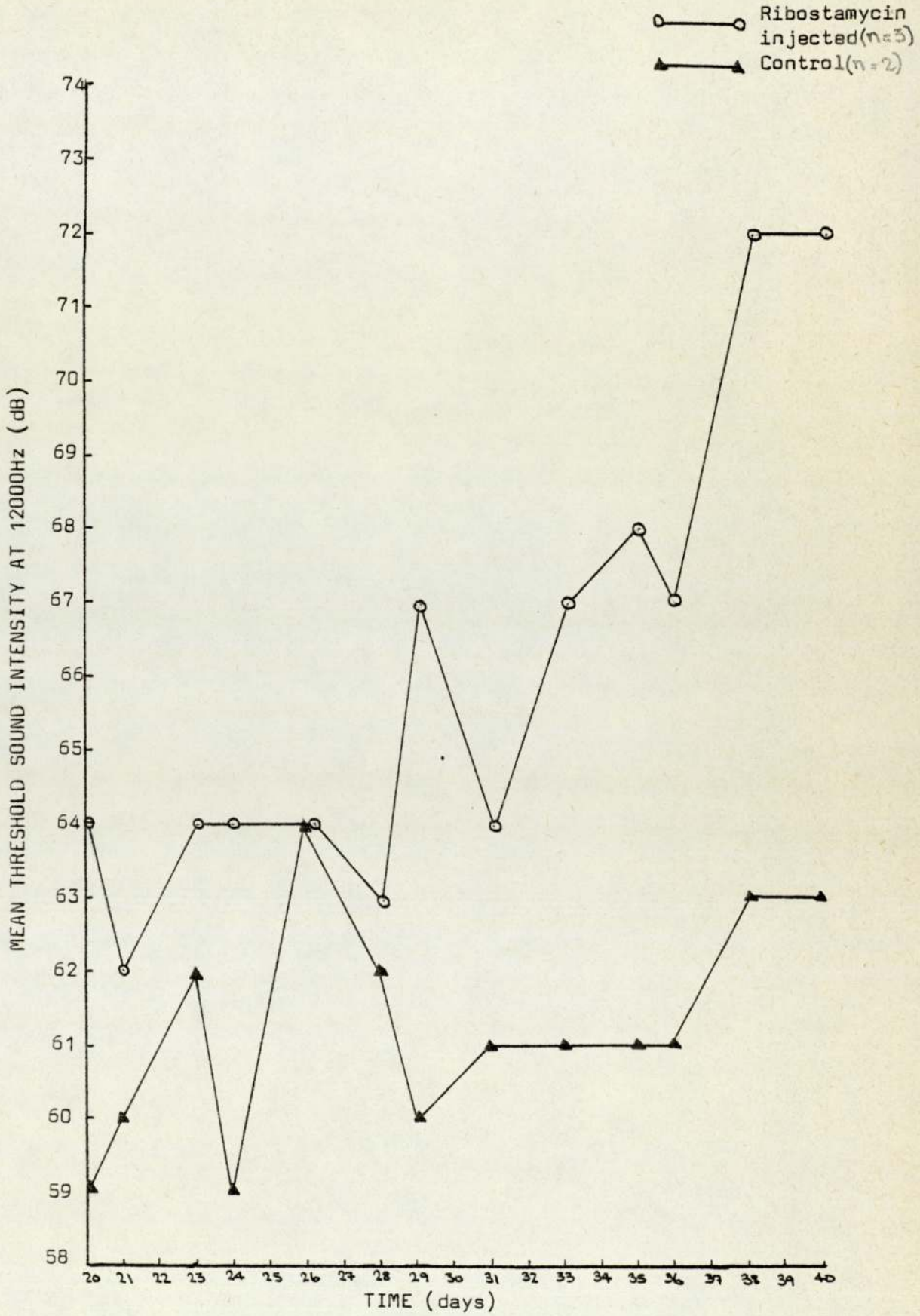


Fig. 42 Most of the outer and inner hair cells look normal. One cell from the first row and one from the second row of the outer hair cells have almost disappeared. Apical turn. Ribostamycin 400 mg/kg/day for 14 days. Survival time - 2 weeks.

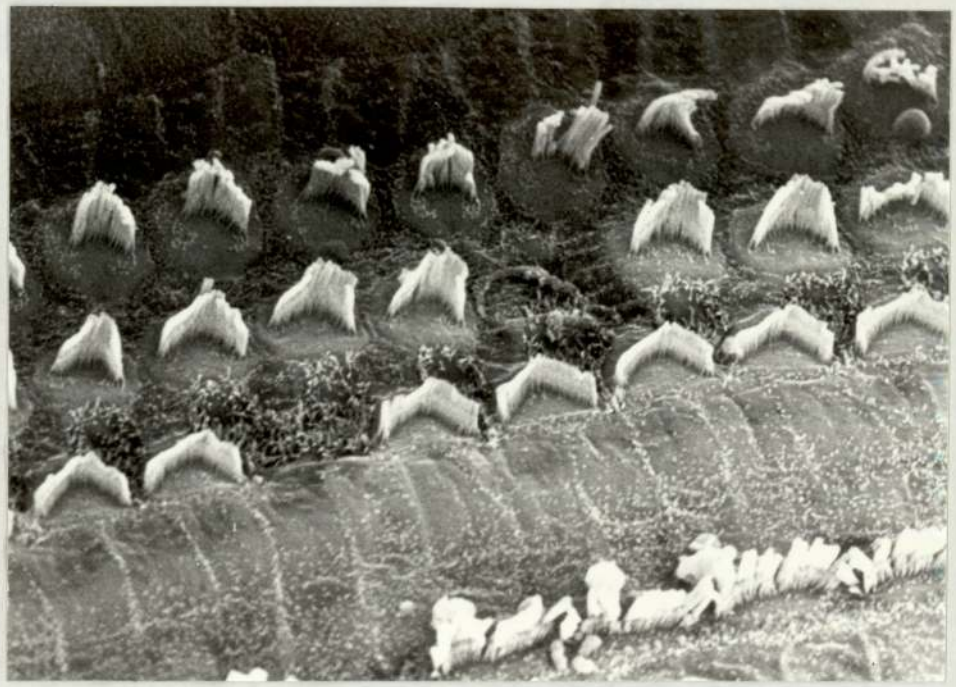


Fig. 43 Cytocochleogram showing scattered missing sensory hair cells (shaded dots) at all turns.

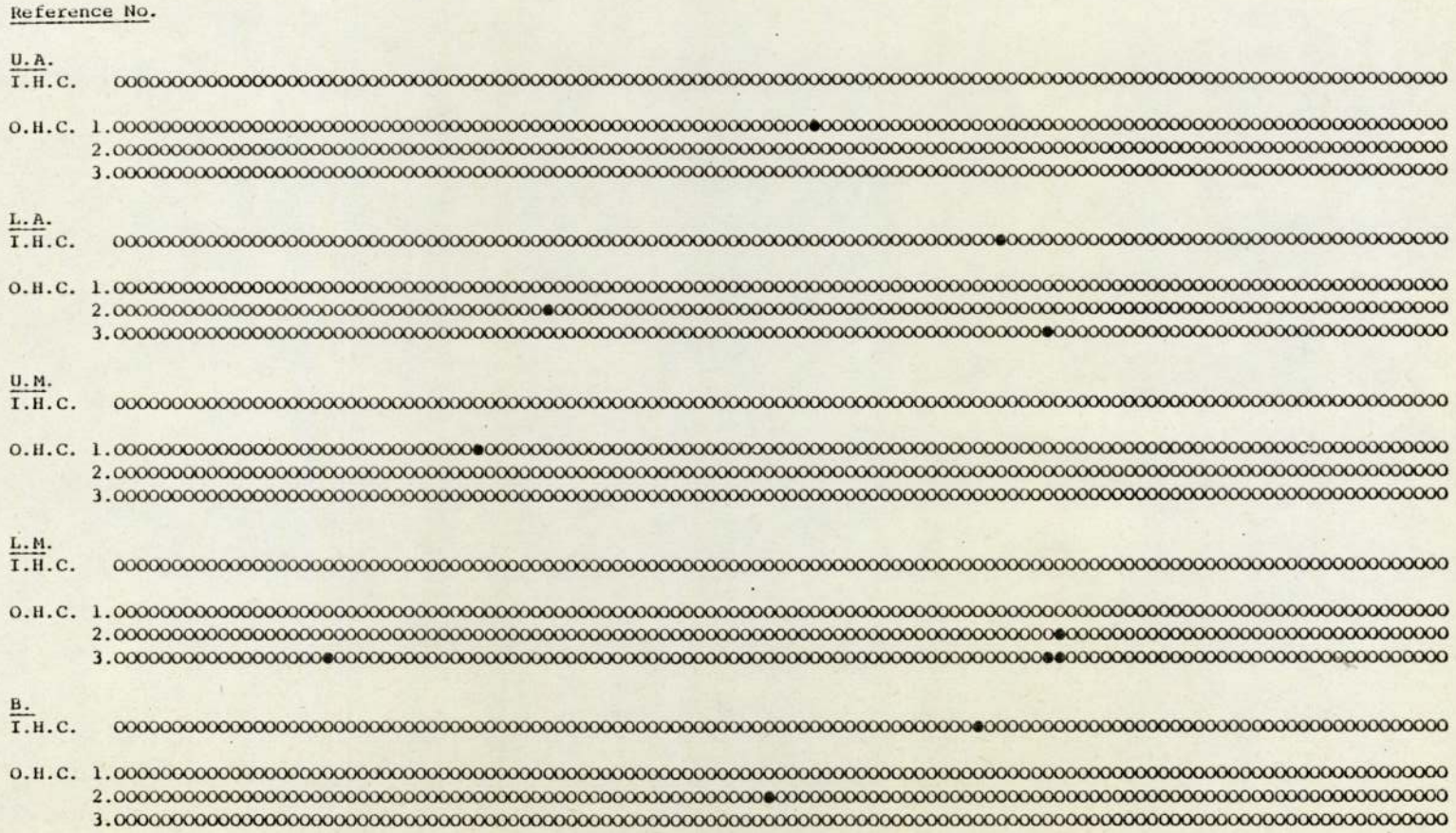


Fig. 44 Normal outer and inner hair cells in the lower apical turn from a control guinea-pig.

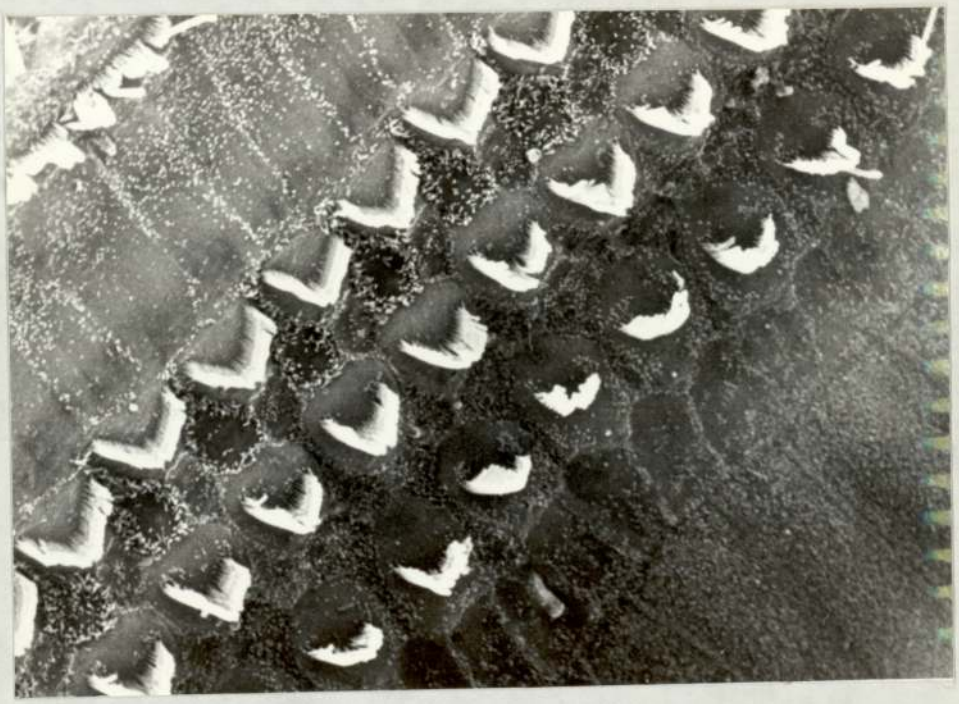


Fig. 45 First row of outer hair cells from a control guinea-pig showing normal surface appearance with a 'W' pattern of arrangement of sensory hairs at high magnification. Apical turn.

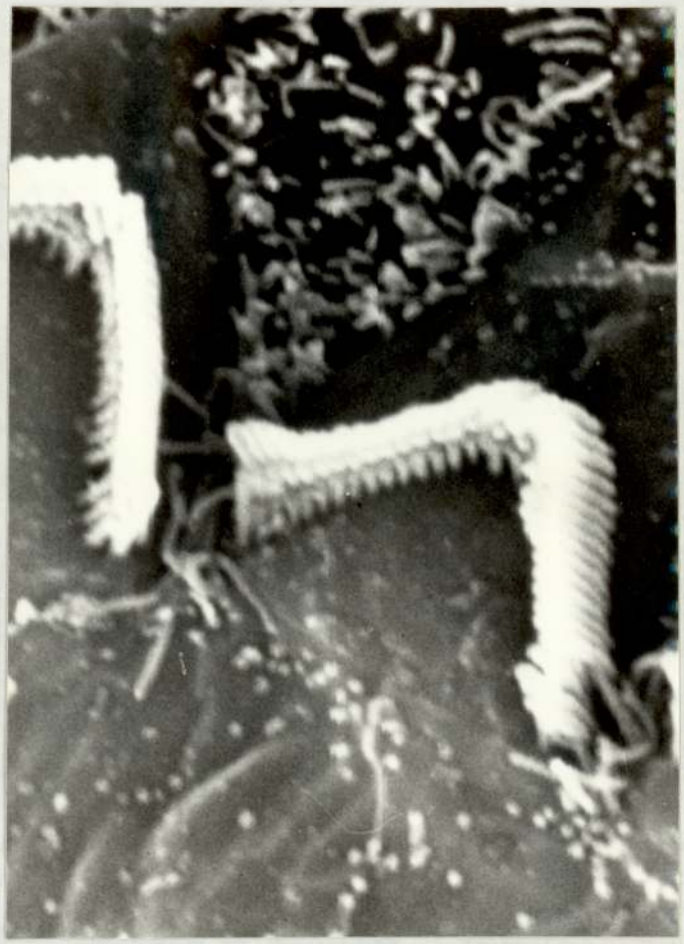


Fig. 46 Outer hair cells showing an irregular 'W' pattern of the sensory hairs in the apical turn. Ribostamycin 400 mg/kg/day for 14 days. Survival time - 4 weeks.

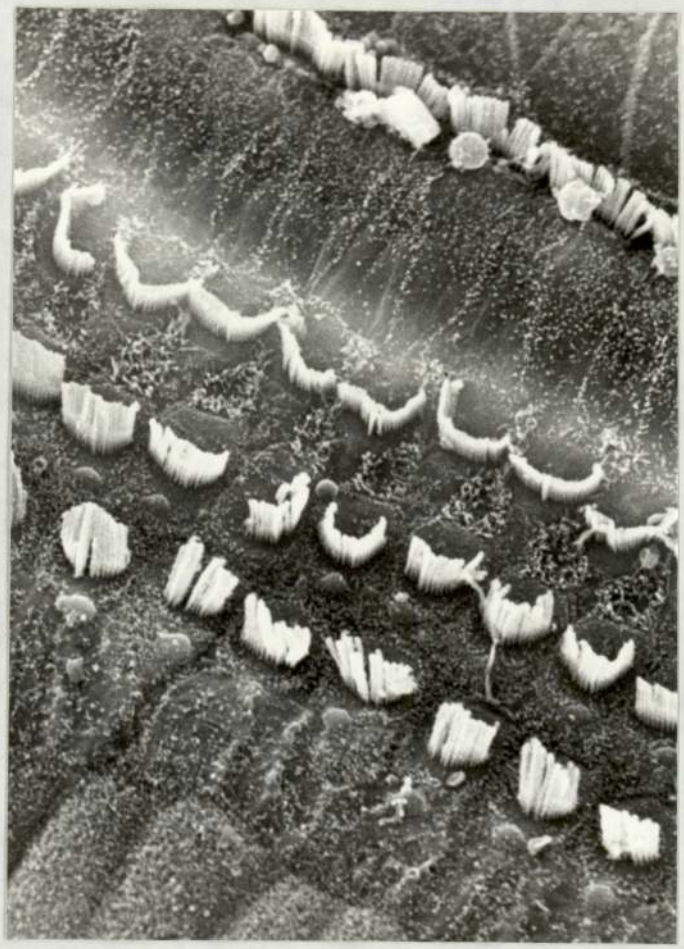
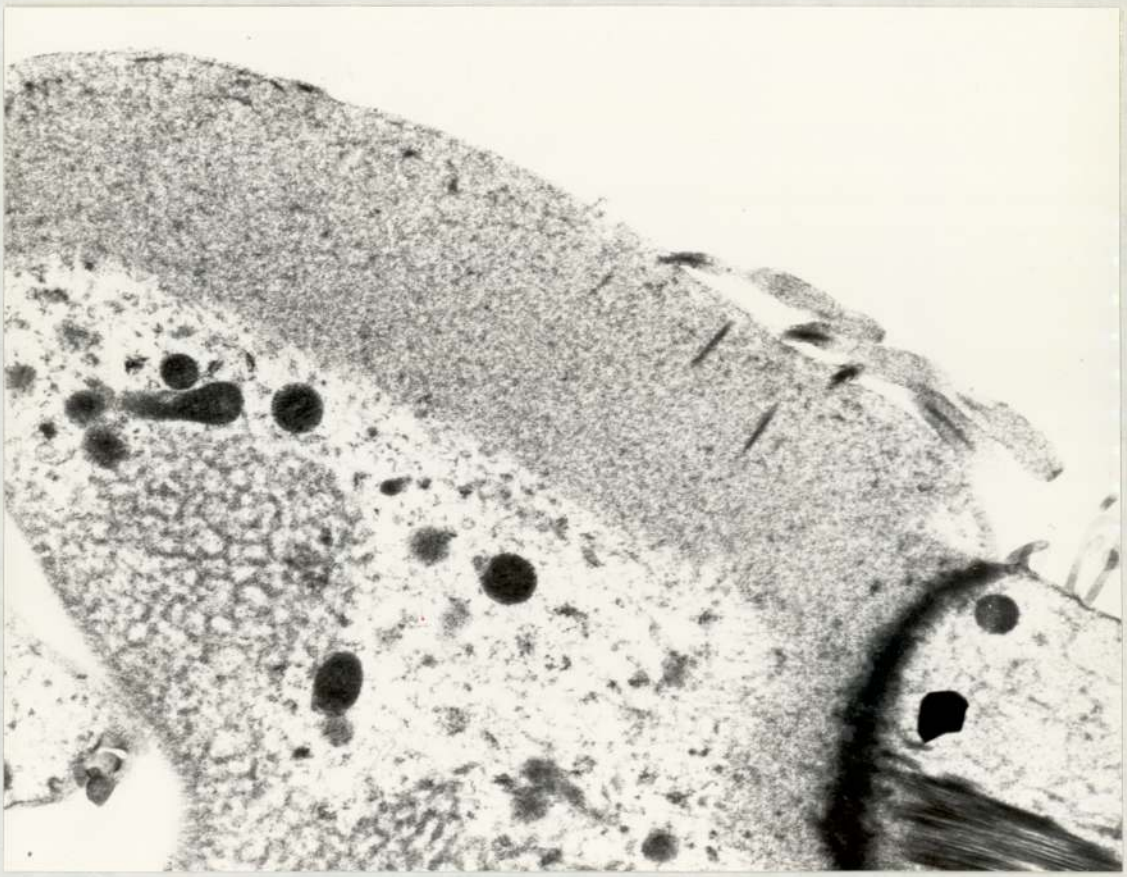


Fig. 47 First row outer hair cell showing disarrangement of sensory hairs. Middle turn.
Ribostamycin 400 mg/kg/day for 14 days.
Survival time - 4 weeks (x14000).



for 2 and 4 weeks after the last dose of ribostamycin. However, this disarrangement of the sensory hairs was not observed in the inner hair cells (Fig. 48). All the animals administered ribostamycin for 14 days (400 mg/day) in the chronic phase showed mitochondrial damage. Disintegration of the internal membranes of the mitochondria was observed in both the outer hair cells as well as the inner hair cells (Fig. 49). These changes did not affect all mitochondria in the cells at the same time, for well preserved mitochondria could also be seen. However, the control group of the guinea-pigs did not show any damage either in the outer hair cells (Fig. 50) or the inner hair cells (Fig. 51).

Fig. 48 Inner hair cell showing normal arrangement of the sensory hairs but some mitochondrial disruption was observed in the supranuclear region. Middle turn.

Ribostamycin 400 mg/kg/day for 14 days.

Survival time - 4 weeks (19,500).

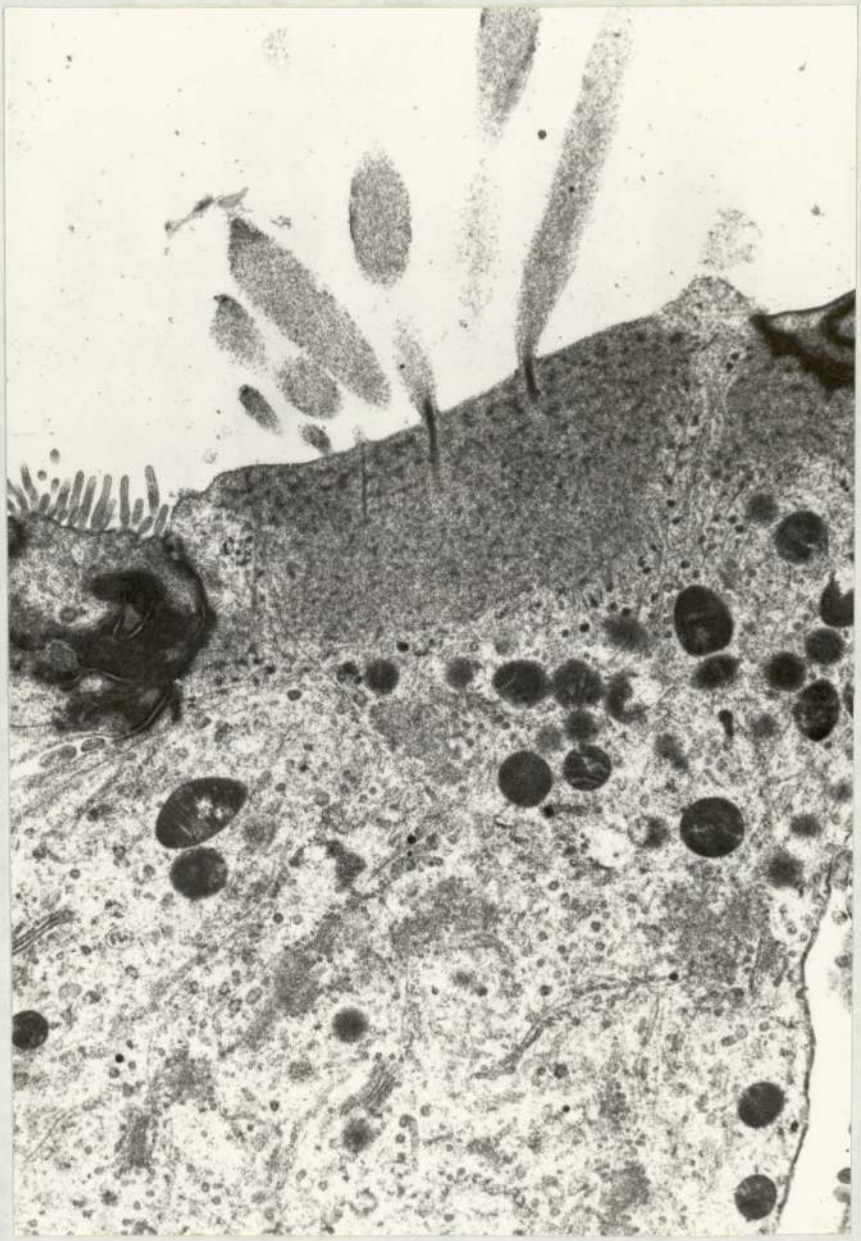


Fig. 49 Base of an outer hair cell showing mitochondrial disruption. Middle turn.
Ribostamycin 400 mg/kg/day for 14 days.
Survival time - 4 weeks (x14,000).

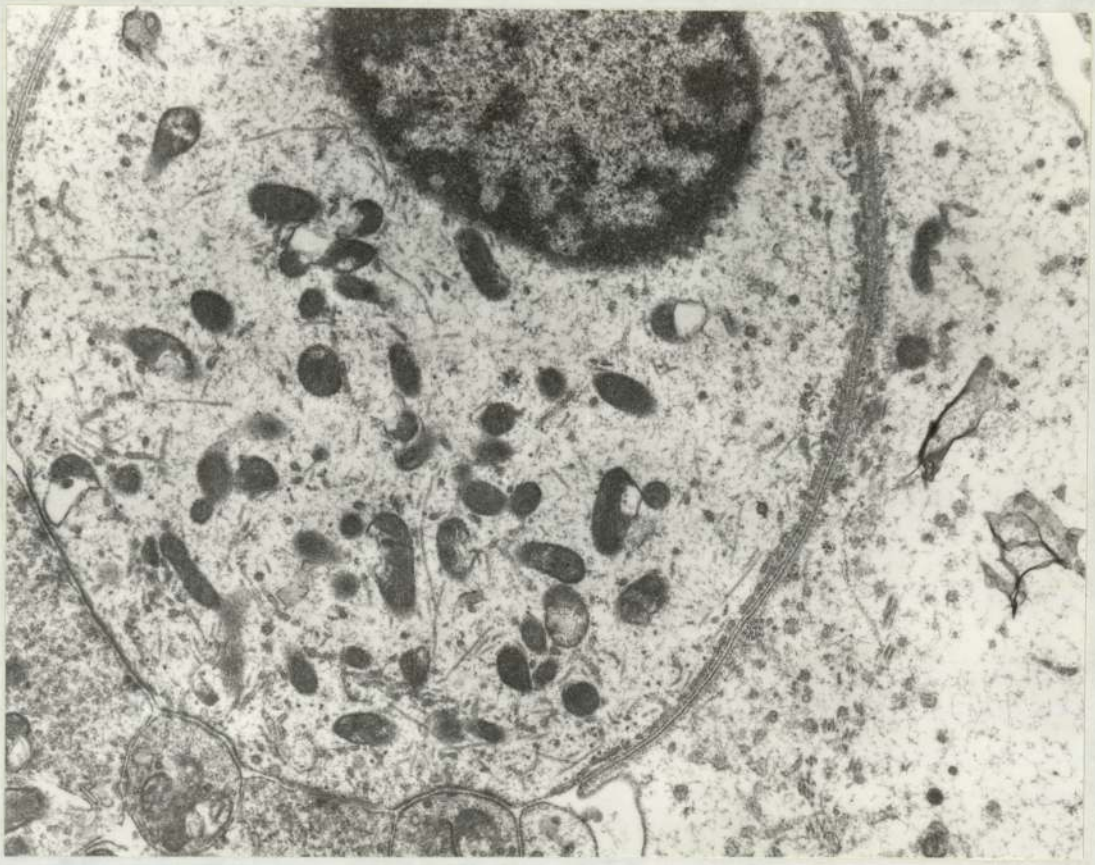


Fig. 50 Base of an outer hair cell from a control guinea-pig showing no mitochondrial disruption (x14,500).

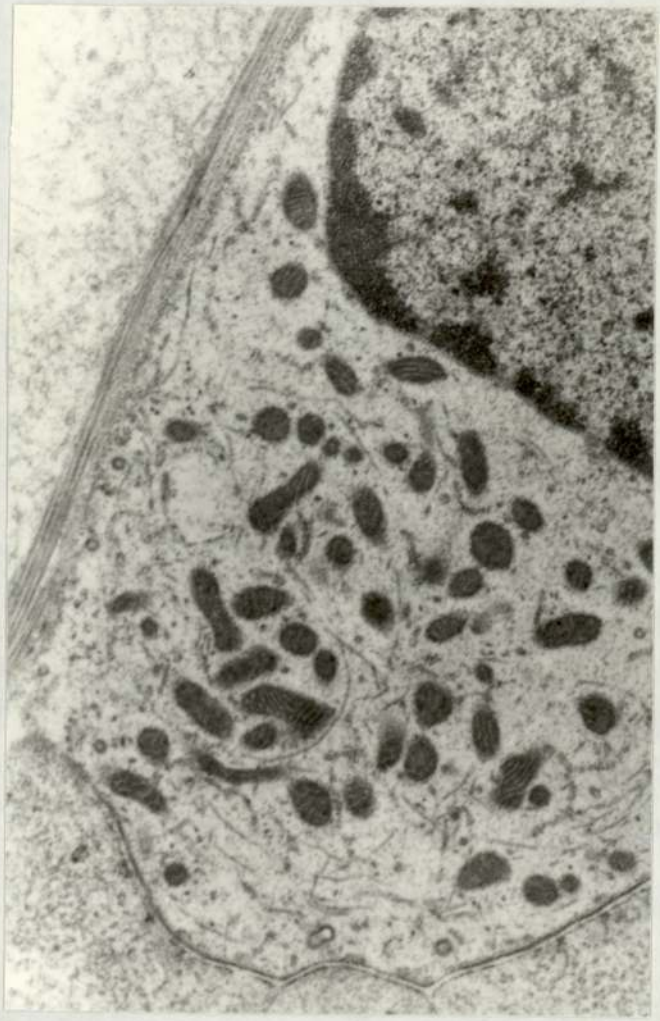
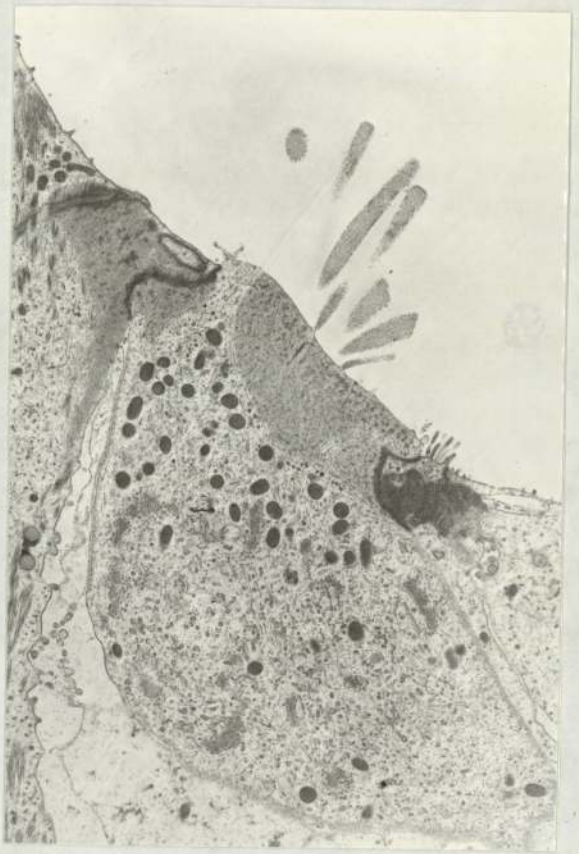


Fig. 51 Inner hair cell from a control guinea-pig showing no ultrastructural damage (x10,250).



8.4 Discussion and Conclusions

After a single subcutaneous injection of 400 mg/kg of ribostamycin, concentrations in serum peaked at 30 min in the guinea-pigs. The peak concentration produced was 592 µg/ml. The rate of excretion was such that 24 h after administration, it had been completely cleared from the serum. However, in the perilymph the drug increased slowly peaking between 2 to 8 h after the injection. The drug was retained in the inner ear for a long time after it had been cleared from the serum. Aminoglycoside antibiotics have been shown to reach the inner ear by passive filtration from the blood (Stupp et al. 1973). However, these antibiotics are eliminated from the inner ear slowly and with difficulty namely by passive transport. Stupp et al. (1973) regarded the inner ear as a 'sieve' that retains and collects the toxic antibiotics while allowing water and other substances to pass through .

The dose-response graph showed that after injection of various doses of ribostamycin from 100 to 400 mg/kg bodyweight, a linear increase in perilymph concentrations occurred with increasing dose. Thus, the steady increase showed that there was no critical serum level for ribostamycin before it can penetrate the inner ear. Stupp et al. (1973) examined the relation between the applied dose of kanamycin, the blood serum level and the perilymph concentrations. A linear relation between the dose and the blood level was observed. In contrast to this, the inner ear levels showed proportionally greater increases with increasing dose above a 25 mg/kg dose. This feature was designated by them as the toxic threshold of the inner ear. They suggested that in

order to avoid exceeding this threshold, either the dose or the corresponding blood level should be kept low.

Histopathological studies of the kidney showed an increased incidence of single necrotic cells and tubular degeneration in the 30 min, 1 h and 2 h groups. Elevation in BUN was also observed in animals from 4 h onwards whereas the serum creatinine levels remained unchanged throughout.

Microscopical investigation of the cochlear tissues from the acute study did not demonstrate hair cell loss or any ultrastructural damage.

Long term dosing with ribostamycin (14 days) produced antibiotic levels in the inner ear that were no different from those found after a single injection. Multiple-dosing, therefore, did not result in accumulation of ribostamycin in the perilymph. In serum, the peak levels were rapidly reached at 15 min and the drug was again excreted rapidly over the first 4 h with just detectable levels (0.3 μg/ml) at 24 h. This pharmacokinetic profile was similar to that following a single dose of 400 mg/kg.

Microscopical studies on the kidneys indicated some focal proximal tubular degeneration and interstitial nephritis in animals killed at 4 and 8 h after the last dose of ribostamycin but not in animals killed after ¼, ½, 1, or 2 h after the last dose of ribostamycin. These pathological changes were less severe in animals killed 12 and 24 h after the last injection. However, these changes were not seen in animals killed after two and four weeks indicating that the renal changes observed in the animals at 4 and 8 h were only minor changes. These changes were reversible as soon

as the drug was stopped.

During 14 days of administration of ribostamycin, no change in hearing threshold was observed in any of the animal groups. However, 15 days after ribostamycin had been stopped, increases in threshold were observed at all frequencies in the drug-injected group. These changes were more pronounced at 12000Hz frequency giving a shift of 10dB compared with the initial test which had been performed on the first day. The control group animals did not show any changes.

Histopathological changes in the cochlea resulting from ribostamycin administration, showed that the sensory cells of the organ of Corti were the elements primarily affected by this drug. The outer hair cells were found to be more vulnerable than the inner hair cells. The first pathologic phenomenon observed in cochleas of animals allowed to survive two or four weeks after the last dose of ribostamycin, was disarrangement of the pattern of sensory hairs on individual outer hair cells at all coils. This effect was most pronounced in the first row of the outer hair cells. In the normal control animals the 'W' formed by the hairs always showed a uniform conformation, but in the degenerating cells, the 'W' figure became an irregular line with hairs often pointing in different directions. This maybe due to the softening of the normally dense cuticular plates of the cells, allowing movement of the rootlets of the individual hairs in the cuticle.

Scattered losses of outer hair cells were also observed in all the specimens. 0.2% of the cells were missing in the control group of animals while the highest loss of cells (0.6%) was recorded in animals allowed to survive two weeks after

the last dose. The missing cells were unevenly distributed in different coils and different rows of the organ of Corti, no definite pattern in the distribution being apparent.

The inner hair cells were found to be more resistant to these drug-induced changes. Only occasionally were there any missing inner hair cells observed. The most pronounced change found with transmission electron microscopy was degenerating mitochondria with disintegrating cristae in both the outer and inner hair cells. Deformation of sensory hairs was observed in animals allowed to survive two or four weeks after the last injection of ribostamycin. Most of the hairs were found to be in close contact with each other probably fused together. This fusion of hairs probably occurs in various stages starting with a decrease in the distance between neighbouring sensory hairs, followed by contacts at certain points, with ultimate disappearance of the membranes between the hairs. It can be inferred that this fusion is due to damage to a factor which normally keeps the hairs apart and prevents them fusing. Spoendlin (1968) demonstrated that the sensory hairs are coated with mucopolysaccharides and it is likely that the 'fuzz' that normally projects as thin strands from the sensory hairs is composed of such mucopolysaccharides. The changes in the membranes causing fusion could depend on the direct incorporation of the antibiotic into the surface membrane, thus changing the properties of the membrane.

From these studies it can be concluded that ribostamycin appears to be less ototoxic and nephrotoxic than the other aminoglycosides. However, further studies would have to

be conducted using other aminoglycosides before one can definitely say that it is less toxic. This study does, however, agree with the studies conducted by the Japanese workers which demonstrated that ribostamycin had no ototoxicity (Harada, 1972) or nephrotoxicity (Kawagishi et al., 1972).

SECTION 9

GENERAL DISCUSSION

Gentamicin-induced ototoxicity has been studied (Section 4, Page 91). From this work, it is apparent that gentamicin is ototoxic. Ototoxicity, both auditory and vestibular, secondary to aminoglycosides has been previously reported (Jackson and Arcieri, 1971; Nordström et al., 1973; Black et al., 1973; Fee, Vierra and Lathrop, 1978; Bender et al., 1979). In general, ototoxicity is considered to be irreversible, but detection of early signs and discontinuation of the drug may permit significant reversion of the acute abnormalities. A general estimate of the incidence of overt ototoxicity in a retrospective study, in which a large number of the case histories of the patients were reviewed, was reported to be about 2% with gentamicin (Jackson and Arcieri, 1971). With more sensitive tests and prospective studies, the incidence of gentamicin ototoxicity may be three to five times as high (Bender et al., 1979).

It is helpful to know the incidence of gentamicin-induced ototoxicity under general conditions of use. In the present study, this appears to be about 8.6%. Figures of overall incidence are not meaningful unless the importance of variations among patients and different features of the therapeutic regimen are known. Toxicity has been suggested to be the result of high serum gentamicin concentrations (Jackson and Arcieri, 1971). The critical level in the production of ototoxicity is unknown. A single high peak level of gentamicin in serum need not cause damage, but sustained moderately high levels can. There is a broad individual variation in the

serum levels attained in relation to the dose (Riff and Jackson, 1971). For prevention of ototoxicity during maintenance therapy, levels of gentamicin in serum as well as renal function, should be monitored. Fear of ototoxicity may cause some patients to be undertreated for susceptible, life-threatening infections with resultant high fatality (Jackson and Riff, 1971). If the drug is essential, the risk of toxicity should not be a basis for denying the patient its therapeutic benefit.

The findings in the present study (Section 4, Page 91) does not justify any conclusions to be drawn concerning the risk factors since the number of patients studied were very small. The patients considered to have incurred gentamicin-induced auditory losses, did not differ from the remainder of the patients in the gentamicin-treated group. The risk of side-effects due to gentamicin did not appear to be increased in patients previously treated with ototoxic drugs. Fifteen patients in the gentamicin-treated group had an existing hearing loss before gentamicin was administered. One of these fifteen patients developed a further hearing loss during gentamicin therapy. Presence of renal insufficiency does not predispose to ototoxicity if the doses are adjusted accordingly.

It is suggested that renal function should be monitored frequently in patients given aminoglycoside antibiotics. Many patients may demonstrate deterioration of glomerular filtration after four to six days which would then lead to high serum aminoglycoside concentrations.

Transient audiometric changes were observed in some of patients in the control group. The importance of interpretation

of audiometric results from very ill patients is stressed. None of the previous studies by other workers (Black et al., 1976; Fee, Vierra and Lathrop, 1978; Bender et al., 1979) had incorporated a control group of patients for comparison of the audiometric responses between the treated and the untreated patients. In these studies, audiometry was performed only at the beginning and at the end of aminoglycoside therapy. The present study has clearly shown that a test at the beginning and at the end of treatment may well show auditory changes (it may be improvement) but only regular audiometric testing will permit progressive changes to be detected.

Some workers had considered that a hearing loss as small as 10dB to represent changes related to therapy (Smith et al., 1977; Panwalker et al., 1978) although a significant reduction in physiological terms is usually greater than 25dB. A change as small as 10dB cannot possibly be attributed to the drug since such changes can occur in normal subjects as well when these tests are performed. This was clearly seen in the present study when normal subjects were tested (Section 3, Page 45). The requirement for a 20dB increase in hearing threshold in order to qualify as auditory toxicity was chosen in the present study to compensate for any variability in the audiograms arising from tests conducted on the ward rather than the sound-proof booth.

Serial hot caloric test was evaluated for monitoring the potential aminoglycoside ototoxicity (Section 2, Page 19). Before applying it on patients, both between-subject and within-subject, between-test variation was assessed by applying

the test on 25 normal subjects. Results showed that a significant reduction in the maximum slow phase velocity occurred in both ears for the group as a whole between Tests 1 and 2. This reduction was sustained at Test 3 three months later. For the maximum frequency, on the other hand, only the right ear responses were reduced at Test 2 and both ears produced equal or greater responses at Test 3 compared to Test 1. Sustained reductions in response were observed in some individuals indicating that this test would not be useful in detecting aminoglycoside vestibulotoxicity.

Protein binding was considered to be conributory factor to ototoxicity. By applying a number of methods (Section 6, Page 147) it was found that as long as the conditions applied in the experiments were controlled, all the methods gave the same results. The amount of binding observed was too low to be of clinical importance.

The effects of a number of fixatives on the ultrastructure of the organ of Corti were studied (Section 7, Page 187). Results showed that micrographs of the ultrastructural studies should be interpreted with caution since artefacts due to tissue preparation procedures can be introduced. This would make the interpretation of the results from drug-damaged organ of Corti difficult.

Ribostamycin, which has been reported to be relatively non-toxic, was also studied. The pharmacokinetics of this drug were studied in both the perilymph and the serum of the guinea-pigs (Section 8, Page 205) to relate it to its toxicity. From these animal studies, it was concluded that ribostamycin did not appear to be ototoxic or nephrotoxic. However, further

studies would have to be conducted, probably on other species, to substantiate these findings. The results of the present study were in agreement with the studies conducted by other workers who demonstrated that ribostamycin had no ototoxicity (Harada, 1972) or nephrotoxicity (Kawagishi et al., 1972).

Finally, it is suggested that workers studying aminoglycoside ototoxicity should include a comparable group of control patients to examine the reliability of the techniques employed to detect this toxicity.

APPENDIX 1

Computer Programme used for the analysis of results of the patients in the Clinical Ototoxicity of gentamicin study.

```
                                    Listing of :SHP7108.MINIG
      MASTER OTOPLOTTER
      REAL ZL(12,6),ZR(12,6),ZBR(12,4),ZBL(12,4)
      DIMENSION IL(12,6),IR(12,6),IBL(12,4),IBR(12,4)
      DIMENSION NAME(15)
      REAL X(12)/1.0,2.0,3.0,4.0,5.0,6.0,7.0,8.0,9.0,10.0,11.0,12.0/
      CALL OPENGINOGP
      READ(1,9) NN
    9 FORMAT(I0)
      CALL UNITS(0.3)
      CALL DEVPAP(100.0+NN*300.0,820.0,0)
      CALL CHASIZ(6.0,9.0)
      CALL AXIPOS(1,30.0,30.0,300.0,1)
      CALL AXISCA(2,12.0,0.0,12.0,1)
      DO 101 I1=1,NN
      READ(1,10)(ITRL,ITEST,(NAME(J),J=1,15))
   10 FORMAT(I4,I6,15A4)
      WRITE(2,12)(ITRL,ITEST,(NAME(J),J=1,15))
   12 FORMAT(1H ,I8,I8,15A4)
      IF(II.GT.1)CALL SHIFT2(380.0,0.0)
      CALL AXIDRA(1.0,1)
      DO 90 IA=1,ITEST
      READ(1,20)((IL(IA,IB),IB=1,6),(IBL(IA,IB),IB=1,4),
     1(IR(IA,IB),IB=1,6),(IBR(IA,IB),IB=1,4))
   13 FORMAT(1H ,6I6)
   14 FORMAT(1H ,4I6)
   20 FORMAT(10I0,/,10I0)
      WRITE(2,13)(IL(IA,J),J=1,6)
      WRITE(2,13)(IR(IA,J),J=1,6)
      WRITE(2,14)(IBL(IA,J),J=1,4)
      WRITE(2,14)(IBR(IA,J),J=1,4)
      DO 80 IB=1,6
      ZL(IA,IB)=-60.0
      ZR(IA,IB)=-60.0
      IF(IL(IA,IB).NE.200)ZL(IA,IB)=FLOAT(IL(1,IB)-IL(IA,IB))
      IF(IR(IA,IB).NE.200)ZR(IA,IB)=FLOAT(IR(1,IB)-IR(IA,IB))
      WRITE(2,15)ZL(IA,IB),ZR(IA,IB)
   15 FORMAT(1H ,2F6.1)
   80 CONTINUE
      DO 70 IB=1,4
      ZBL(IA,IB)=-60.0
      ZBR(IA,IB)=-60.0
      IF(IBL(IA,IB).NE.200)ZBL(IA,IB)=FLOAT(IBL(1,IB)-IBL(IA,IB))
      IF(IBR(IA,IB).NE.200)ZBR(IA,IB)=FLOAT(IBR(1,IB)-IBR(IA,IB))
      WRITE(2,15)ZBL(IA,IB),ZBR(IA,IB)
   70 CONTINUE
   90 CONTINUE
      DO 60 J=1,6
      CALL AXIPOS(1,30.0,30.0+(J-1)*110,90.0,2)
      CALL AXISCA(2,9,-60.0,30.0,2)
      CALL AXIDRA(-1,-1,2)
      CALL GRASYM(X,ZL(1,J),ITEST,3.0)
      CALL GRAPOL(X,ZL(1,J),ITEST)
```

Appendix 1 Continued .....

```
      CALL PENSEL(2,0.0,0)
      CALL GRASYM(X,ZR(1,J),ITEST,4.0)
      CALL GRAPOL(X,ZR(1,J),ITEST)
      IF(J.LT.2.OR.J.GT.5)GOTO 58
      CALL PENSEL(5,0.0,0)
      CALL GRASYM(Z,ZBL(1,J-1),ITEST,1,0)
      CALL GRAPOL(X,ZBL(1,J-1),ITEST)
      CALL PENSEL(7,0.0,0)
      CALL GRASYM(X,ZBR(1,J-1),ITEST,2.0)
      CALL GRAPOL(X,ZBR(1,J-1),ITEST)
   58 CALL PENSEL(1,0.0,0)
      CALL DASHED(1,10.0,5.0,5.0)
      CALL MOVTO2(30.0,70.0+(J-1)*110)
      CALL LINBY2(300.0,0.0)
      CALL MOVBY2(0.0,40.0)
      CALL LINBY2(-300.0,0.0)
      CALL MOVBY2(5.0,10.0)
      CALL CHAHOL(12HFREQUENCY *.)
      CALL CHAINT(J,2)
      CALL BROKEN(0)
   60 CONTINUE
      CALL MOVTO2(50.0,695.0)
      CALL CHAARR(NAME,15,4)
      CALL MOVTO2(50.0,680.0)
      CALL PENSEL(5,0.0,0)
      CALL SYMBOL(1)
      CALL CHAHOL(16H LEFT BONE     *.)
      CALL PENSEL(7,0.0,0)
      CALL SYMBOL(2)
      CALL CHAHOL(17H RIGHT BONE     *.)
      CALL PENSEL(1,0.0,0)
      CALL SYMBOL(3)
      CALL CHAHOL(18H LEFT AURAL      *.)
      CALL PENSEL(2,0.0,0)
      CALL SYMBOL(4)
      CALL CHAHOL(14H RIGHT AURAL*.)
      CALL PENSEL(1,0.0,0)
      CALL MOVTO2(120.0,20.0)
      CALL CHAHOL(21H   SUCCESSIVE TESTS*.)
  101 CONTINUE
      CALL DEVEND
      STOP
      END
      FINISH
```

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