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A STUDY OF PENICILLIN CONCENTRATIONS
IN BOVINE CONJUNCTIVAL SAC FLUID
AS IT PERTAINS TO THE TREATMENT
OF MORAXELLA BOVIS INFECTION

A THESIS

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ABSTRACT

Infectious bovine keratoconjunctivitis is one of the commonest eye diseases of cattle. A specialised organism *Moraxella bovis*, is generally held to be responsible for the often serious damage to the cornea and the conjunctiva. Temporary blindness is common but even without treatment, most cattle eventually regain their vision. Although the disease has been recognised for more than 30 years in most cattle-farming areas of the world, only in the past 8-10 years has it become evident in New Zealand.

A wide range of antibacterial products has been used for treatment but there has been very little definitive work undertaken which would form a sound basis for any schedule of medication. In view of the information lacking in this respect, it was decided to study the pharmacokinetics of an antimicrobial drug in the conjunctival sac fluid after different formulations of the drug had been administered by different routes. Penicillin was chosen as the model antibiotic because it is remarkably free of side effects, effective against *M. bovis* and available in a range of products suitable for administration by various means. The overall aim of the work was to determine a concentration-time profile for penicillin in conjunctival sac fluid (CF) and it was reasoned that this data could then be used to establish a schedule of treatment that would produce an optimum effect against *M. bovis*.

The preliminary requirement of the research programme was to

investigate a suitable method of collecting unchanged samples of CF over a number of days (i.e. 1-7 days). As soon as the project was initiated, it became clear that any substantial distress to the animals caused lachrymation, and the possibility of the CF then containing endogenous antibacterial substances could not be discounted. Of the three methods of collection tested, the use of blunted capillary tubes was found to be best for the purpose because the method avoided any local tissue irritation and the cattle soon learned to tolerate any associated handling and minor restraint. Special safeguards were built into the experiment to confirm the absence of antibacterial substances other than penicillin. The specificity of the inhibitory substance in CF, namely penicillin, was regularly confirmed by testing for parallelism against standard dilutions of penicillin, and periodically by neutralizing all antibacterial activity in a sample, using penicillinase.

Estimations of the penicillin concentration in CF samples were carried out by means of the agar-well diffusion technique. Minor modifications to the basic assay system were required to ensure that the sensitivity would cover the range of penicillin concentrations expected to appear in CF. After a series of titrations involving the size of the inoculum of the indicator organism, the depth and volume of the agar medium, the volume of the test solution for each well and the incubation schedule, each variable was standardized for all subsequent assays. A large plate (28 x 28 cm, containing 64 wells 4.5 mm in diameter,

2.5 cm apart) permitted the assay of 12 CF samples alongside four standard dilutions of penicillin under uniform conditions. Using *Bacillus subtilis* as the indicator organism, the assay system was sensitive in the range between 10 and 0.07 iu/ml penicillin, with a standard error of predicted values of 0.04-0.17.

In order to nominate a penicillin concentration in CF that would be effective against *M. bovis*, the penicillin sensitivity of several strains of the organism were measured in terms of their minimum inhibitory concentration (MIC). The four New Zealand isolates tested were highly sensitive; most having a MIC of penicillin of 0.03 iu/ml, and this was identical for the bactericidal concentration. After making allowance for an *in vivo* safety factor of 5, the minimum therapeutic concentration (MTC) of penicillin against *M. bovis* was defined for this series of experiments to be 0.15 iu/ml. The length of time that the penicillin concentration in CF remained equal to or above the MTC following treatment with any particular product, was considered as the duration of therapeutic concentration (DTC; hours) for that particular treatment.

The major experiments using clinically normal cows involved the estimation of penicillin concentration in CF following systemic, subconjunctival or topical administration. Every treatment was repeated in five or six animals but without exception any variation in the DTC between eyes and animals was found to be not significant. In all experiments, the decline in penicillin

concentration in CF followed an exponential pattern, irrespective of the route of administration.

A series of systemic injections was carried out by the appropriate route using three different products of penicillin at a standard dose-rate of 20,000 iu/kg. Penicillin concentrations observed in CF ($\pm$ SEM) following the intravenous injection of sodium benzyl penicillin (peak 2.0 iu/ml and DTC 5.5 ± 0.25 hours) and the intramuscular injection of procaine penicillin (peak 1.0 iu/ml and DTC 16.5 ± 1.25), were considered inadequate for the treatment of IBK.

Penethamate hydriodide, administered by either the intramuscular or the subcutaneous route, achieved an approximate peak concentration of 3.0 iu/ml and produced a minimum therapeutic duration of 61 ± 5.57 hours. Such a difference between the kinetics of penethamate hydriodide and benzyl penicillin was attributed to the greater lipid solubility of the diethylamino-ethyl ester of penicillin. Although the profile of penicillin in CF following penethamate administration seems favourable, the cost of the product would probably prohibit its regular use. In a further experiment in which half the dose was used, the DTC was reduced to 23.5 ± 4 hours.

A subconjunctival injection of procaine penicillin at a dosage of 6×10^5 iu in 2 ml, administered either through the skin or through the conjunctiva, achieved an approximate peak of 8 iu/ml

for both routes and DTCs of 67.6 ± 5.0 hours and 40 ± 2.6 hours respectively. The faster rate of penicillin decay following an injection given through the conjunctiva, is possibly attributable to the back diffusion of the drug through the needle puncture. In general, the penicillin profile in CF following a subconjunctival injection is conducive to an extended bactericidal effect and the trial results tend to confirm the clinical impressions of its usefulness in the field. Treatment by this means is relatively cheap and easily undertaken. If a more prolonged effect is desirable, another dose might be administered two days after the first.

Topical application of sodium benzyl penicillin in aqueous solution at a concentration isotonic with 0.9% saline, produced a DTC in CF for 12.6 ± 1.5 hours. This duration is considered long for a water soluble salt in an aqueous base. When this salt and other less water soluble ones were formulated in an ointment base, the time of effect was significantly prolonged. Sodium benzyl penicillin and procaine penicillin in the ointment base, produced DTCs of 39.8 ± 2 hours and 37 ± 4 hours respectively, while the ointment formulation of benethamine penicillin produced a DTC of 56 ± 4.5 hours. The prolonged decline observed for all eye ointments can be partly accounted for by the viscous nature of the base but other differences may be dependent on the relative water solubility of each penicillin salt. In addition, the various structures of the surface mucosae such as crypts and specialised cells, are likely to retard free diffusion

and therefore retain penicillin, in CF. The extent of dissociation of a substance depending on its pK_a may also influence the overall rate of decline.

Regular examination of the treated eyes and cell counts undertaken on CF samples, did not indicate any inflammatory reaction even after repeated application of ointments.

The various penicillin profiles observed in CF now provide a sound basis for establishing treatment schedules. Optimum treatment schedules can be advocated for different penicillin products, based on concentrations and durations that could be expected to control *M. bovis* infection in superficial tissues of the eye. However, in order to confirm the therapeutic effectiveness of such schedules, controlled clinical trials using infected animals are obligatory.

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PREFACE

Infectious bovine keratoconjunctivitis (IBK) is well known as an economically important eye condition in cattle and it occurs in most farming areas of the world. Outbreaks of the disease were first reported in New Zealand only recently (Anon,1975), but it quickly became established and the condition is now prevalent in cattle throught the country.

The disease is caused by the bacterium *Moraxella bovis* often in association with certain predisposing factors which have been incriminated in spontaneous outbreaks. While the disease is not responsible for a high rate of mortality, and indeed outbreaks are eventually self-limiting, its economic importance stems from unthriftiness in diseased animals and consequent losses in production. In addition, the disease causes disruption to the normal farm routine as blind animals warrant extra attention.

In the long term the use of vaccines seems the most promising approach to control of IBK but products giving a high protection rate are not yet available. Fortunately *M. bovis* is sensitive to a range of commonly used antibacterial drugs and treatment, particularly of early cases, is usually successful.

Effective chemotherapy of IBK demands the maintenance of therapeutic concentrations of the administered drug in fluids covering the bovine eye for sufficient time to eliminate the

infective organism , *M. bovis*. In spite of the variety of chemicals that have been used over the years for that purpose, there is very little information in the literature on drug concentrations obtainable in conjunctival fluids after administration of products by any route.

Accordingly it was decided to undertake investigations in this area; to determine if the route of administration and any specific features of the drug product would influence either the peak concentration obtainable in conjunctival sac fluid or the length of time effective levels could be sustained.

Penicillin may not necessarily be the drug of choice to treat IBK under field conditions, but it was chosen as the model antibiotic in this study for special reasons. These were, its overall safety, its effectiveness against *M. bovis* and in particular, the range of products available containing different salts of penicillin in a form suitable for administration by a number of routes.

In broad terms, this study set out to establish basic information on the pharmacokinetics of penicillin in conjunctival sac fluids. It was hoped that the investigations would lead to a greater understanding of the drug's distribution and such knowledge would enable more rational schedules of treatment to be devised.

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL FEATURES OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

1.1.1 Introduction

Infectious bovine keratoconjunctivitis (IBK) is a very common and a highly contagious eye infection of cattle. The acute stage of the disease is characterized by inflammatory changes in the conjunctivae, ulceration of the cornea with profuse lachrymation, photophobia, spasm of the eyelids and blepharitis (Jackson, 1953; Formston, 1954). The morbidity rate is high. Comprehensive reviews on all aspects of the disease have included more detailed descriptions of the clinical appearance (Barner, 1952; Henson & Grumbles, 1960; Wilcox, 1968; Hughes & Pugh, 1972; Baptista, 1979).

Infectious bovine keratoconjunctivitis was first described in the late nineteenth century by Billings (1889; cit. Barner, 1952). More recently the disease has been identified in most cattle-farming areas of the world (Watt, 1951; Jackson, 1953; Formston, 1954; Gallagher, 1954; Adinarayanan & Singh, 1961; Pedersen, 1970).

In New Zealand the disease became evident in 1973-74 (Cooper, pers. comm.) and the first outbreak was reported in 1975 (Anon, 1975). The absence of any records before this suggests a recent introduction of the disease to New Zealand (Harris *et al*, 1980). The disease is less common in the South Island than in the North Island and there appears

to have been an increasing pattern of outbreaks over the years 1975-79 (Corrin, 1980; Harris *et al*, 1980).

A survey carried out in the North Island has also shown that IBK appears to have been recently introduced into New Zealand and has now become a widespread disease (Sinclair, 1982). The same survey indicates that the annual incidence is still rising; outbreaks following a seasonal pattern and being most evident in the late summer.

The disease is economically important to the cattle industry of New Zealand. The main losses are due to unthriftiness in beef animals (Thrift & Overfield, 1974; Thomas *et al*, 1978) and reduced milk production in dairy herds (Bedford, 1976). Some losses are sustained as a result of deaths associated with misadventure (Sinclair, 1982). An additional impact on economic management may be associated with inefficient grazing, the cost of veterinary services and the disruption caused by the need to handle and treat infected animals (Slatter *et al*, 1982a).

1.1.2 Causative agent

It is now generally accepted that the disease is caused by the bacterium, *Moraxella bovis* (Henson & Grumbles, 1960; Pugh & Hughes, 1972; Chandler *et al*, 1980), and certain predisposing factors such as solar radiation (Kopecky *et al*, 1980; Kopecky *et al*, 1981) have been incriminated. *Moraxella bovis* is an aerobic, capsulated, fimbriated, nonmotile, nonsporing, Gram negative, bipolar staining, short diplobacillus (Henriksen, 1952; Jackson, 1953). The bacterium belongs to the genus

Moraxella; members of which are capable of colonizing and multiplying on the external mucosae of warm blooded animals (Henriksen, 1976).

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Moraxella bovis can be recovered from the conjunctival sac of diseased animals with the use of moistened swabs and culturing these on blood agar plates (Pedersen, 1970). After incubation at 37°C for 24 hours, colonies of *M. bovis* exhibit beta haemolysis (Henriksen, 1952) and can be confirmed by their unique biochemical reactions; alkaline peptonization of litmus milk, proteolysis on Loeffler's serum, production of cytochrome oxidase, nonfermentation of carbohydrates, inability to grow on MacConkey agar and inability to reduce nitrate (Jackson, 1953; Pugh et al, 1966; Wilcox, 1970; Carter, 1976; Fraser & Gilmour, 1979). Certain strains of *M. bovis* are nonhaemolytic and many of these have been isolated from clinically normal eyes (Pugh et al, 1966).

1.1.3 Transmission of the disease

The disease outbreaks are generally prevalent in summer and early autumn (Harris et al, 1980; Webber & Selby, 1981b) and this pattern of disease could be attributed to ocular susceptibility associated with increased solar radiation (Hughes et al, 1965). The mode of natural transmission is believed to be by direct contact (Gallagher, 1954; Pugh & Hughes, 1972). In addition, the face fly *Musca autumnalis* (De Geer) has been reported to act as a mechanical vector (Brown & Adkins, 1972).

Pure cultures of *M. bovis* grown on blood agar plates are capable of producing IBK in cattle when dropped into the undamaged

conjunctival sac (Pugh & Hughes, 1972; Chandler *et al*, 1980; Wilson *et al*, 1981). More consistent results are claimed following the use of ultra-violet radiation on challenged eyes (Hughes & Pugh, 1971; Pugh & Hughes, 1976).

1.1.4 Control of the disease in herds

Segregation of infected animals and the strict quarantine of animals are traditional measures applied to the control of any contagious disease, and the same may be practical for IBK under certain conditions. In addition, control of transmission of the disease by mechanical vectors has been attempted using an insecticide (Beug, 1976; McNutt, 1976) such as cypermethrin (Caballo, 1980).

Under experimental conditions, the use of live vaccines has induced an increase in serum antibodies with increased inoculations and the resistance developed was comparable to that seen in animals developing natural immunity after recovering from IBK (Hughes & Pugh, 1971). Some protection against IBK has been achieved in new-born calves after feeding colostrum from vaccinated cows (Pugh *et al*, 1980). However, the use of either specific ribosomes (Pugh *et al*, 1981) or pili fractions (Pugh & Hughes, 1976) of *M. bovis* in attempts to improve the immunogenicity of the vaccine, was equivocal. It was also found that injecting vaccines into the conjunctiva rather than administering them subcutaneously did not produce significantly better results (Webber & Selby, 1981a). In one study, some reduction in the incidence of IBK was obtained by weekly preventive treatment using furazolidone eye spray (Hughes *et al*, 1979).

Individual animal treatment with antimicrobial products has been widely used for the past thirty years (Pugh, 1978) and it is still considered to be the best approach to control infection in the event of an outbreak. Aspects of antimicrobial treatment will be dealt with in greater detail in the following section.

1.2 TREATMENT OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

1.2.1 Introduction

The mild form of IBK involves the inflammation of both cornea and conjunctiva, causing little damage. Animals often recover in 3-6 weeks without any treatment being administered (Pugh, 1978). As a result, IBK has been described as a self-limiting disease (Bedford, 1976). However, severely affected eyes in which the changes include extensive ulceration may take many months before resolution is complete and corneal scars may persist. Early antibacterial treatment of the disease is beneficial, because *M. bovis* infection is cleared and further development of the disease is stopped. Accordingly, there is no serious damage to the cornea (Formston, 1954) and control of *M. bovis* prevents further spread of the disease. It is well recognized that at the advanced stages of the disease in which there is severe corneal ulceration, antibacterial treatment has only a limited effect on the process of resolution (Cooper, 1960).

Although specific treatment of IBK requires the use of antibacterial products, supportive surgical measures have been adopted in the U.S.A. and the U.K. (Anderson et al, 1976; Dalton, 1976). For instance, the *membrana nictitans* has been drawn across the cornea

and sutured to the lateral conjunctival fornix thereby forming a conjunctival flap (Anderson *et al*, 1976). It has been suggested that this surgical measure helps to improve the effectiveness of medicines by delaying drug elimination from the conjunctival fluids and in addition it protects the cornea against further damage.

In spite of the obvious advantages associated with individual animal treatment and the significant economic impact of the disease, only 11% of New Zealand farmers treat their cattle for IBK (Sinclair, 1982). In contrast, a survey in Australia has shown that 86% of dairy herds, 38% of beef herds and 80% of mixed-type herds are routinely treated (Slatter *et al*, 1982a). The frequency of treatment ranged from either three times daily to once weekly, or it was limited to a single dose when the disease was first observed. Those farmers who were reluctant to treat IBK cases, believed either that the disease did not warrant attention (Slatter *et al*, 1982a) or that there were insufficient benefits from medication (Sinclair, 1982). The failure to treat by some farmers has been attributed to ignorance of the widespread nature of the disease and the effect it might have on production. Furthermore, the rationale for any particular treatment schedule is not widely understood. However farmers who routinely treated their animals, have reported satisfactory cure rates and other benefits associated with a quick recovery (Slatter *et al*, 1982a).

The results of a recent survey revealed that homidium bromide (27%), oxytetracycline (23%), chloramphenicol derivatives (14%)

and penicillin derivatives (14%) were the drugs most frequently used by Australian cattle producers (Slatter *et al*, 1982a). The latter two were rated highly for their effectiveness.

1.2.2 Treatment schedules and drugs used

Systemic routes of administration:

Different combinations of antimicrobial substances, sometimes including a corticosteroid, have been used occasionally through one of the systemic routes of administration. For example, intravenous injections of sulfamethazine at a dose rate of 100mg/kg, have been used to eliminate the carrier state and to avoid any subsequent recrudescence of the disease (Pugh, 1978).

When two dose rates of oxytetracycline were used intramuscularly for the treatment of experimentally induced IBK, some lesions resolved after the use of the higher dose rate (11 mg/kg). Uncured eyes were treated effectively with tylosin at 5 mg/kg and at 7 mg/kg; the higher dosage brought about a higher rate of recovery (Pugh & McDonald, 1977). The same authors found that a mixture of penicillin and dihydrostreptomycin given intramuscularly, used in combination with topically applied nitrofurazone powder, was also successful. In another study (Pugh *et al*, 1982) the prophylactic use of tylosin by intramuscular injection in cows before parturition, failed to prevent the transmission of *M. bovis* infection to their offspring.

Subconjunctival injections:

This route of administration of drugs is believed to maintain

therapeutic activity in ocular tissues for an extended period (Leopold, 1964; Slatter, 1981). It is therefore being widely used in treating IBK in New Zealand (Cooper, pers. comm.), and overseas (Table 1.1).

Topical instillations:

A wide range of medicaments including antiseptics and antimicrobial substances are used topically under field conditions in the treatment of IBK (Table 1.2). The effectiveness of most products is unproven.

Slow release mechanisms:

In treating IBK under field conditions, the frequency with which treatment can be administered is believed to be insufficient to maintain therapeutic drug concentrations (Slatter et al, 1982a). Thus, there is a general interest in the use of slow release devices that are retained in the conjunctival sac for an optimum period and that do not cause undue reactions.

For example, a 7/32 inch diameter eye pellet consisting of oxytetracycline (5 mg), polymyxin B sulphate (10,000 iu) and tetracaine hydrochloride (1 mg) was reported to be successful in treating IBK without causing any side effects (Hawley, 1954). A residual tetracycline concentration of 0.67 microgram/ml was noted in tears, 31 hours after application.

A ring device made up of polyvinyl tubing has been satisfactorily retained in the bovine eye (Hughes & Pugh, 1975) but prolonged retention was accompanied by increased tear and mucous secretion.

The incorporation of an antibiotic into this device would enable it to be used therapeutically.

Various collagen inserts of thickness ranging from 0.075 to 0.125 mm were also found to be easily inserted into the conjunctival sac. These have been retained for periods of up to eight days without causing signs of irritation (Slatter *et al*, 1982c). The rate of release of gentamicin from such a collagen insert produced relatively low concentrations during the first few hours but after eight to nine hours a steady state concentration of 1 microgram/ml was achieved and the drug was gradually eliminated over 24 hours.

Hydrophilic contact lenses with 75% water content have also been developed specifically for the bovine eye. Retention over four days in the bovine eye has been accompanied by ocular irritation, corneal opacity and local oedema (Slatter *et al*, 1982b).

An apparatus for subpalpebral lavage was constructed in order to treat a case of IBK. Complete restoration of vision was attained within two weeks after frequent infusion of gentamicin ophthalmic solution (Nyack & Padmore, 1982).

1.3 SENSITIVITY OF *MORAXELLA BOVIS* TO ANTIBACTERIAL SUBSTANCES

Most *M. bovis* isolates are susceptible to a range of commonly used antibacterial substances. However, to obtain optimum results, the *in vitro* sensitivity of a single isolate should be determined (Pugh & McDonald, 1977) and a range of methods are available for use.

The disc diffusion technique is routinely used to investigate the sensitivity of recently isolated strains of bacteria and the numerous results concerning *M. bovis*, are listed in Table 1.3. In particular, *M. bovis* was repeatedly reported to be highly sensitive to penicillin when evaluated by the disc diffusion technique (Barner, 1952; Henriksen, 1952; Kliwer & Gee, 1960; Pedersen, 1970; Wilcox, 1970). The tube dilution technique is a more laborious method but is occasionally used in sensitivity testing. Chloramphenicol was fractionally more effective than Aureomycin in studies using the tube dilution technique (Gallagher, 1954).

By definition, the minimum inhibitory concentration (MIC) of an antibacterial substance is the lowest concentration at which the test organism does not produce any visible opacity after incubation (Cruickshank *et al*, 1975). The test to determine the MIC of an antibacterial substance against a bacterium, although more complicated than other routine sensitivity tests, provides a quantitative measurement of the *in vitro* susceptibility. Use of the microdilution technique in MIC studies on *M. bovis* has been reported by Webber *et al* (1982) who undertook a comprehensive programme to investigate a number of commonly used antimicrobial agents against different isolates of *M. bovis*. The sensitivities of numerous *M. bovis* strains were not significantly different one from another, although they were obtained from field outbreaks over a wide geographic area in Missouri and over a period of years. In addition, haemolytic isolates had higher MIC values than nonhaemolytic isolates against gentamicin, penicillin and streptomycin. However, *M. bovis* constantly showed some resistance to

cloxacillin (Wilcox, 1970; Webber *et al*, 1982) at 1 microgram/ml. Subsequent studies of Buswell *et al* (1982) showed that the MIC of cloxacillin against *M. bovis* ranged between 0.31 and 2.5 microgram/ml.

1.4 PENICILLIN CONCENTRATION IN SUPERFICIAL TISSUES OF THE EYE AFTER DIFFERENT METHODS OF ADMINISTRATION

1.4.1 Introduction

The specific treatment of IBK necessitates the maintenance of an adequate concentration of a given antibiotic at the site of infection, namely the cornea and the conjunctiva. Many different antimicrobial substances have been used by various routes in an attempt to attain optimum concentrations in the extraocular tissues. In particular, penicillin is widely used for IBK (section 1.2) as many isolates of the causative organism *M. bovis* are highly sensitive to the antibiotic (section 1.3). The nontoxic nature of the drug to farm animals even at higher dosages and the nonirritant nature of the antibiotic on eye tissues, account for its popularity.

1.4.2 Systemic injections of penicillin to produce concentrations in the conjunctival sac fluid

The writer was unable to find any references that dealt with penicillin concentrations in the bovine eye following single dose treatment. References to other species of animal are limited too, apart from certain exceptions, and much of the work referred to was undertaken more than 20 years ago. The only available information on penicillin excretion in bovine tears was presented by Pedersen (1973). In those experiments he maintained a constant plasma concentration of either benzyl penicillin or penethamate hydriodide by continuous intravenous

infusion, and thereafter assayed penicillin appearing in the conjunctival sac fluids. For benzyl penicillin he achieved a tear:plasma concentration ratio of 0.002:1 and 0.003:1. In a similar experiment using penethamate hydriodide, the corresponding ratio ranged from 1.9:1 to 2.1:1 (i.e. a drug concentration in tears which is approximately twice as high as the concentration in plasma). The same study demonstrated that the ratio was not influenced either by the plasma concentration *per se* or by the rate of tear secretion. The higher tear:plasma ratio that resulted from the use of penethamate hydriodide could be explained by the largely unionized nature of the diethylaminoethyl ester molecules of penicillin in plasma. They would be more inclined to accumulate in tears which are found to have a pH slightly lower than that of blood, $7.29 \pm .003$ as against $7.49 \pm .10$ respectively.

The intravenous injection of 50,000 iu 'pure penicillin' into rabbits achieved a concentration of 1.0 iu of penicillin in the cornea after 30 minutes (Sorsby & Ungar, 1946). Similar injections of 25,000 iu resulted in a concentration of 0.5 iu penicillin by $2\frac{1}{2}$ hours after the injection.

After intravenous injection of 12,800 iu/kg into dogs, Struble & Bellows (1944) found that penetration of soluble penicillin into the eye tissues had occurred within 15 minutes. Drug concentrations in the cornea were lower than those of the sclera and conjunctiva. By three hours after administration, traces were still detectable in the extraocular fluid although the blood was completely free of penicillin.

It was shown that 10,000 i_u of penethamate hydriodide injected into rabbits intramuscularly achieved on average, 0.12 iu penicillin/gram of wet tissue of the cornea (Bleeker & Mass, 1958).

1.4.3 The concentrations produced following subconjunctival injections

The subconjunctival route is often preferred in ophthalmological practice because of the traditional belief in its duration of effect and it does appear to be effective in the management of acute eye infections (Table 1.1). However, patient apprehension, pain, inconvenience, subsequent inflammation and expense have all been offered as limitations (Gelatt, 1968; Havener, 1974).

Two methods of subconjunctival injection have been described. One involves the piercing of the conjunctiva, while in the other, the skin of the eyelid is pierced leaving the conjunctiva intact (Wine *et al*, 1964; Havener, 1974; Gay, 1981; Sinclair *et al*, 1981). When using the conjunctival route, leakage of deposited drug back on to the eye through the needle puncture has been recorded (Wine *et al*, 1964) and the same study suggested that the drug could be partly lost through the lymphatic and vascular circulations.

Oakley *et al* (1976) estimated the concentration of penicillin in the cornea of rabbits after subconjunctival injection of soluble benzyl penicillin. This study showed that there was a graded concentration from the periphery towards the centre of the cornea (Fig. 1.1); such a concentration gradient supports the view that drug distribution takes place through the corneoscleral limbus.

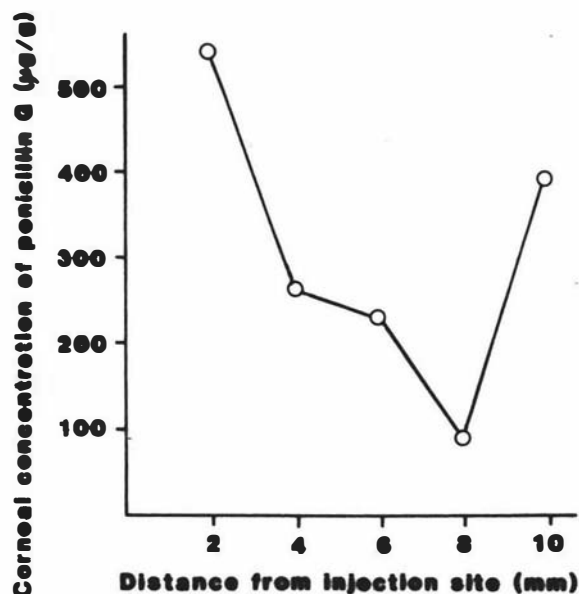


Fig.1.1 Intracorneal distribution of penicillin G, $\frac{1}{2}$ hour after subconjunctival injection of 50 mg (Oakley *et al*, 1976).

Absorption of penicillin from the subconjunctival space into the general circulation has been shown on several occasions. For instance, a subconjunctival injection of a dose of 25,000 iu of penicillin into both eyes of rabbits followed by arterial blood analyses, produced concentrations of 2.0, 0.25, 0.125, 0.06 iu/ml and a trace, at 1, 2, 3, 3 $\frac{1}{2}$ and 4 hours respectively (Sorsby & Ungar, 1946). A subconjunctival injection of twice the above dose in rabbits has produced a peak blood concentration of 3.7 iu/ml within $\frac{1}{2}$ hour (Andrews, 1947).

1.4.4 The concentrations produced following topical instillations

Simplicity of treatment without the need for injection or patient apprehension has made topical instillation a widely acceptable method of treating eye diseases (Gellat, 1968; Brightman, 1980). Penicillin is often used by this route in the treatment of IBK in cattle (Table 1.2).

Penicillin in distilled water at concentrations between 20,000 and 50,000 iu/ml is considered isotonic with 0.9% saline and therefore it is indicated for topical instillation (Sorsby & Ungar, 1946). Such isotonic preparations are nonirritant to the superficial tissues of the eye (Struble & Bellows, 1944; Sorsby & Ungar, 1946; Sorsby, 1960).

Treatment by topical instillation causes very high drug concentrations initially on the surface of the eye. This is followed by a rapid phase of decline due both to absorption through the conjunctiva and cornea (Havener, 1974) and to elimination via the nasolachrymal duct (Janes & Stiles, 1963). Elimination of instilled solution from the conjunctival sac has been shown to follow first-order kinetics and the rate of decline is proportional to the viscosity of the instilled solution (Chrai *et al*, 1973).

It is believed that increased viscosity prolongs ocular contact and thereby increases the drug absorption (Havener, 1974; Brightman, 1980; Maurice, 1980). In order to improve the duration of action, penicillin is often incorporated into an ointment base. Instillation of 0.1 gram of ophthalmic ointment containing 50,000 iu/gram penicillin into the eyes of rabbits has produced corneal concentrations of 2.0, 0.75, and 0.25 iu/ml at 1, 2 and 4 hours respectively (Sorsby & Ungar, 1946). A similar ointment at one tenth the strength has been recommended for human use (McWilliam, 1972).

Some current work undertaken in the U.K. at the same time as the Massey work was being evaluated, demonstrated that a single topical instillation of 125 mg benzathine cloxacillin in an oily base had maintained a

therapeutic concentration (4.01 ± 3.3 microgram/ml) against *M. bovis* for approximately 56 hours on the surface of the bovine eye (Buswell *et al*, 1982).

1.5 SUMMARY

Infectious bovine keratoconjunctivitis is caused by *M. bovis* and today the disease is economically important in New Zealand. This disease is controlled using chemotherapy with the objective of maintaining sufficiently high concentrations of antibacterial substance at the site of infection.

Moraxella bovis is sensitive to a range of antibacterial substances. Penicillin has been shown to play an important role in arresting the infection. Penicillin is regularly used in veterinary practice to treat IBK, although the scientific literature does not provide suitable evidence to substantiate its use by any route of administration.

Table 1.1 Use of drugs by the subconjunctival route in the treatment of infectious bovine keratoconjunctivitis

Drug	Formulation	Treatment schedule	Comments made by the author(s)	Reference
chloramphenicol	20% aqueous solution	0.74 - 1.5 ml single injection or once in 6 days	responded within 3 days and "cornea healed within 21 days"	Fleming, 1975
combination of ampicillin benzathine penicillin) procaine penicillin) triamcinolone acetonide atropine	200 mg/ml 150,000 iu/ml 2 mg/ml 0.5 gram/ml	2-3 ml of the combination		Murray, 1976
methyl prednisolone penicillin with dihydrostreptomycin	0.5 ml (20 mg/ml) Depomedrol 0.5ml (20,000 iu/ml (0.25 gram/ml Distrycillin	single treatment	responded within 7 days	Schrimsher, 1970
aqueous procaine penicillin aqueous ampicillin	Mylipen Penbritin	2 ml	"single injection was effective in 160 eyes, some of which had been infected up to 3 weeks"	James, 1976
penicillin/streptomycin	Streptopen	2 ml	immediate & substantial drop in the prevalence of <i>M. bovis</i> was noted	Sinclair, 1982
procaine penicillin benzathine penicillin	Duplocillin	1-2 ml single treatment	improved within 2-6 days and only few cases required second injection	Cryer, 1976

Table 1.2 Use of drugs by topical application in the treatment of infectious bovine keratoconjunctivitis

Drug	Formulation	Treatment schedule	Comments made by author(s)	Reference
(a) <u>Antiseptics and antimicrobial substances:</u>				
zinc sulphate	2% solution	used at the initial stages	"effective as antibiotics"	Faull & Hawksley, 1954
cyanide of mercury hyperchloride of mercury arsenical preparations	1:4000 solution) 1:1000 solution)	2 or 3 times daily at initial stages	effective in treating IBK	Brown, 1934
silver nitrate	1.5% solution		found to be of benefit in preventing the disease in new born calves	Brown, 1934 Pugh, 1978
boric acid or mercuric chloride followed by Metaphen or Mercurophen	1:2000 solution		Possibility of this treatment was indicated only in dairy cows	Klussendorf, 1952
	Furacin powder (Eaton) Topazone (Eaton)		"given good results"	Beug, 1976
ethidium bromide	0.5% eye ointment or aqueous solution	once or twice daily for 5 consecutive days	equally effective as other anti- biotics at the initial stages; healing proceeded quickly without toxic effects	Cooper, 1960
tylosin tartrate	aqueous solution 50 mg/ml spray	twice daily for 3 consecutive days	"all but corneal opacity and ulceration disappeared within 5 days"	Ellis & Barnes, 1961
tylosin-neomycin	ophthalmic powder	once or twice daily for 3 days	effective in treating IBK	Sampson & Gregory, 1974

Table 1.2 continued

Drug	Formulation	Treatment schedule	Comments made by author(s)	Reference
chloramphenicol	ophthalmic solution	2.5 mg instilled twice daily, at initial stages	effective in clearing visual signs of the disease within 2 - 3 days.	Faull & Hawksley, 1954
chloramphenicol	1% ophthalmic ointment	single dose	early infection arrested in 24 hours, repeated application required for advanced stages	Gallagher, 1954 Jackson, 1954
	ointment	once daily for 3 consecutive days twice daily		Pugh, 1978 Nyack & Padmore, 1982
sulfathiazole	5% powder		given beneficial results	Pugh, 1978
combination of chloramphenicol, urea, isopropyl alcohol, propylene glycol, methyl violet & propellants	aerosol		"handy aerosol container" & effective in treatment	Anon, 1961
gentamicin	eye spray	prophylactic use		Staples, Kotta & Smith, 1981
Penicillin	intramammary preparation	50,000 iu instilled twice daily	effective at initial stages of infection, responded within 2 - 3 days	Faull & Hawksley, 1954
(b) <u>Miscellaneous compounds</u>				
cod liver oil		twice daily	"did not alter the course of infection"	Faull & Hawksley, 1954
atropine	eye ointment		given good results	Beug, 1976
physiological saline & foreign protein		injection	given good results	Beug, 1976

Table 1.2 continued

Drug	Formulation	Treatment schedule	Comments made by author(s)	Reference
warm physiological saline with tincture of opium	5 minims/ounce		beneficial on ulcerated cornea	Brown, 1934
thyrotropin releasing hormone	0.01% aqueous solution	twice daily for 6 consecutive days	opacity was cleared without toxicity	Kato & Ono, 1981
cortisone acetate	powder or 0.5% suspension		improved	Scott, 1957

Table 1.3 Sensitivity of *Moraxella bovis* towards antibacterial substances as determined by a disc diffusion technique

Antimicrobial substance	Disc potency	Sensitivity	Number of isolates tested	Reference
ampicillin	10 microgram	69.4% sensitive	160	Pugh & McDonald, 1977
	10 microgram	sensitive	84	Webber, Fales & Selby, 1982
	2 microgram	100% sensitive	25	Wilcox, 1970
bacitracin	2,10 & 20 iu	sensitive	4	Barner, 1952
	2 iu	95% sensitive	160	Pugh & McDonald, 1977
	10 iu	sensitive	84	Webber, Fales & Selby, 1982
chloramphenicol		sensitive		Arora & Killinger, 1976
	10, 30, 60 iu	sensitive	4	Barner, 1952
		sensitive	10	Pedersen, 1970
	5 microgram	98.1% sensitive	156	Pugh & McDonald, 1977
	30 microgram	sensitive	84	Webber, Fales & Selby, 1982
	10 microgram	100% sensitive	25	Wilcox, 1970
cloxacillin	10 microgram	100% sensitive	25	Cullinane, pers. comm.
	1 microgram	100% resistant	84	Webber, Fales & Selby, 1982
	5 microgram	52 % resistant	25	Wilcox, 1970
erythromycin		sensitive		Arora & Killinger, 1976
	2 microgram	94.4% sensitive	160	Pugh & McDonald, 1977
	10 microgram	100 % sensitive	25	Wilcox, 1970

Table 1.3. continued

Antimicrobial substance	Disc potency	Sensitivity	Number of isolates tested	Reference
furazolidone	200 microgram	100% sensitive	25	Cullinane, pers. comm.
gentamicin	10 microgram	sensitive	84	Webber, Fales & Selby, 1982
kanamycin	30 microgram	97.5% sensitive	160	Pugh & McDonald, 1977
	5 microgram	sensitive	84	Webber, Fales & Selby, 1982
lincomycin	2 microgram	75 % resistant	160	Pugh & McDonald, 1977
miconazole	1 microgram	100% sensitive	12	Van Cutsem, Van Gerven, & De Keyser, 1983
neomycin		sensitive		Arora & Killinger, 1976
		sensitive	10	Pedersen, 1970
	5 microgram	96.9% sensitive	160	Pugh & McDonald, 1977
	30 microgram	sensitive	84	Webber, Fales & Selby, 1982
nitrofurantoin	50 microgram	97.5% sensitive	160	Pugh & McDonald, 1977

Table 1.3 continued

Antimicrobial substance	Disc potency	Sensitivity	Number of isolates tested	Reference
nitrofurazone	100 microgram	sensitive	84	Webber, Fales & Selby, 1982
oxytetracycline	10, 30, 60 microgram	sensitive	4	Barner, 1952
	5 microgram	76.9% sensitive	160	Pugh & McDonald, 1977
	30 microgram	sensitive	84	Webber, Fales & Selby, 1982
penicillin		sensitive		Arora & Killinger, 1976
	.5, 1.0, 10 iu	sensitive	4	Barner, 1952
	2 iu	68 % sensitive	25	Cullinane, pers. comm.
		sensitive	10	Pedersen, 1970
	10 iu	60.6% sensitive	160	Pugh & McDonald, 1977
	10 iu	sensitive	84	Webber, Fales & Selby, 1982
	1.5 iu	100 % sensitive	25	Wilcox, 1970
polymyxin B		sensitive		Arora & Killinger, 1976
		sensitive	10	Pedersen, 1970
	300 iu	sensitive	84	Webber, Fales & Selby, 1982

Table 1.3 continued

Antimicrobial substance	Disc potency	Sensitivity	Number of isolates tested	Reference
streptomycin		resistant	5	Arora & Killinger, 1976
	10 microgram	80 % sensitive	25	Cullinane, pers. comm.
		sensitive	10	Pedersen, 1970
	10 microgram	68 % resistant	66	Webber, Fales & Selby, 1982
	10 microgram	100% sensitive	25	Wilcox, 1970
dihydrostreptomycin	.5, 1, 10 microgram	'least sensitive'	4	Barner, 1952
	10 microgram	77.5% sensitive	160	Pugh & McDonald, 1977
sulphamethoxypyridiazine	150 microgram	64.4% resistant	160	Pugh & McDonald, 1977
sulphonamides		sensitive	10	Pedersen, 1970
triple sulphonamide	300 microgram	sensitive	84	Webber Fales & Selby, 1982
sulphafurazole	100 microgram	80% resistant	25	Wilcox, 1970

Table 1.3 continued

Antimicrobial substance	Disc potency	Sensitivity	Number of isolates tested	Reference
tetracycline		sensitive		Arora & Killinger, 1976
	10 mg	100 % sensitive	25	Cullinane, pers. comm.
		sensitive	10	Pedersen, 1970
	5 microgram	85 % sensitive	160	Pugh & McDonald, 1977
	10 microgram	100 % sensitive	25	Wilcox, 1970
tylosin	15 microgram	100 % sensitive	4	Pugh & McDonald, 1977

Table 1.4 Antibiotic concentrations in the extraocular tissues after penicillin administration by subconjunctival injection

Product	Dosage	Species	Penicillin concentration in the cornea or conjunctiva time (hours)					Comments made by the author(s)	Reference
			$\frac{1}{2}$	1	2	3	4		
penicillin	20,000 iu	rabbits		>30 iu/g		0.8 iu/g		higher dosage has resulted in longer duration of action	Sorsby & Ungar, 1947
	50,000 iu	rabbits	97 iu/g	76 iu/g	35 iu/g	18.75 iu/g	4.87 iu/g		
sodium penicillin	50,000 iu in 0.5 ml saline	rabbits	167 iu/g	39.3 iu/g	5.4 iu/g		1.7 iu/g	"economical in skilled hands"	Andrews, 1947
sodium penicillin	0.5 ml of solution containing 5,000 iu/ml	rabbits		53.2 iu/g	34.9 iu/g		traces	results were not readily interpreted for other species	von Sallmann, 1945
penicillin	2,500 iu in 0.25 ml of normal saline	rabbits	(highest level observed in the cornea was 28 iu/g (highest level observed in the conjunctiva was (106-449 iu/g and was not detectable after 3 hours					highest tolerable strength	Struble & Bellows, 1944
penicillin	25,000 or 50,000 iu in 0.5 ml water	rabbits	concentration in cornea was .25 iu/ml at 5 hours					dosage was well tolerated	Sorsby & Ungar, 1946
Potassium penicillin G	0.25 ml containing 50 mg	rabbit	concentration in cornea was 139 microgram/g in 15 minutes					significant levels were achieved in all areas of the cornea	Oakley, Weeks & Ellis, 1976

CHAPTER 2

ESTIMATION OF PENICILLIN CONCENTRATION IN CONJUNCTIVAL SAC FLUID USING A BIOASSAY TECHNIQUE

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CHAPTER 2

ESTIMATION OF PENICILLIN CONCENTRATION IN CONJUNCTIVAL SAC FLUID USING A BIOASSAY TECHNIQUE

INTRODUCTION

A bioassay may be defined as an estimation of the relative potency of an unpurified drug by comparing its biochemical, pharmacological or toxic effects with a highly purified preparation of the same drug (Fingl & Woodbury, 1975). When equivalent doses of the standard preparation and the unknown sample produce the same response in the same range, the dose-response curves constructed can be used to estimate directly the drug concentration of the unknown sample.

For investigating materials in which the antibiotic concentration requires to be estimated, modifications of the basic bioassay technique have included the cup plate method (Foster & Woodruff, 1944; Grove & Randall, 1955) and the agar-well diffusion method (Bennett *et al*, 1966; Litwack *et al*, 1969). These two, with various minor modifications have been used extensively for assaying a range of antibiotics present in clinical specimens.

The agar-well diffusion method which was ultimately selected for use in this study involved seeding an agar plate with an appropriate indicator organism, filling precut wells in the agar with the samples of either unknown concentrations or the standard

dilutions, and after incubation observing the zone of growth inhibition around the wells.

Numerous variables are encountered in bioassay techniques (Lees & Tootill, 1955) and major ones fall into groups associated with the indicator organism, preparation of the assay plate and time schedule of incubation.

The bioassay technique requires that the end point measured must not be influenced by the solvent in which the standard dilutions of active material are prepared (Bennett *et al*, 1966). Bovine CF is only available in very small quantities and therefore the suitability of other solvents for preparing standard dilution series of penicillin had to be investigated.

MATERIALS AND METHODS

The assay method, which had to be specially modified for small volumes of specimens, has been described by Lees & Tootill (1955), Kavanagh (1963), Bennett *et al* (1966) and Simon & Yin (1970).

2.1 ROUTINE BIOASSAY TECHNIQUE

2.1.1. Indicator organism

In preliminary experiments, the minimum inhibitory concentration of penicillin against *M. bovis* had been determined and details of these investigations appear later in Chapter 4. After making allowance for a safety factor to ensure a therapeutic concentration in the conjunctival sac fluid (CF), it was decided that *Bacillus*

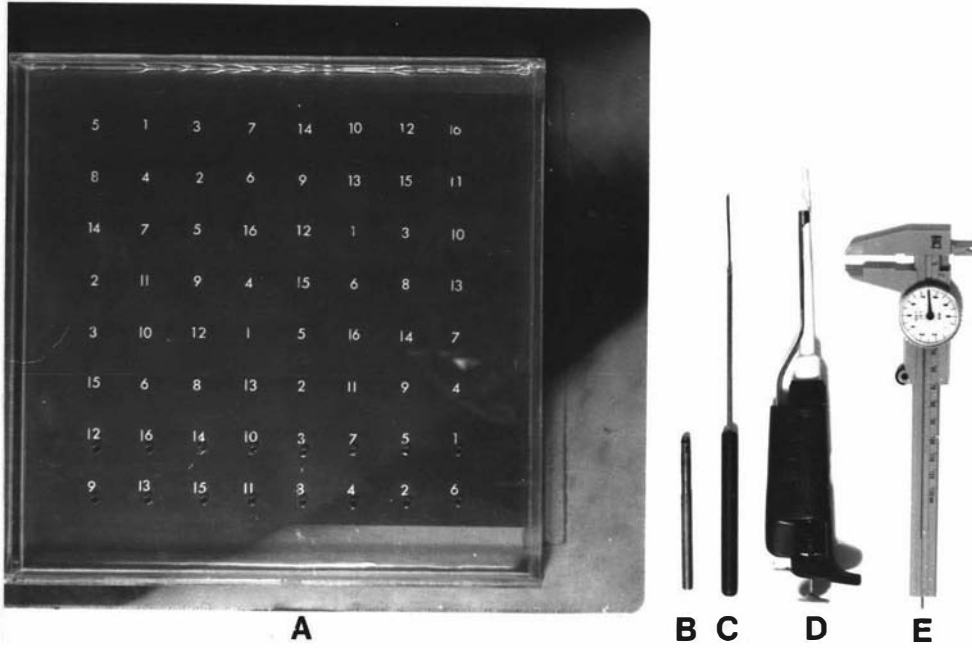


Plate 2.1 Materials used for bioassay

- A. assay plate placed over the paper guide
- B. tube borer
- C. needle
- D. adjustable micropipette
- E. vernier calliper

subtilis would be the most appropriate indicator organism to cover the range of concentrations of interest (Foster & Woodruff, 1944).

A preparation of *B. subtilis* spore suspension[§] was stored at 4°C and before use diluted 1:200 in distilled water to obtain the working strength of suspension.

2.1.2 Preparation of the assay plate

The assay medium (Appendix 2.1) was prepared by adding 0.5 ml of diluted spore suspension to 200 ml of melted agar held at 60°C. The inoculated agar medium was well mixed and poured into square glass plates, 28 x 28 cm in dimension. The plates containing the cooling agar were left uncovered for approximately 5 minutes before having aluminium lids applied. Poured plates were left on the bench for a further 30 minutes and then transferred to storage at 4°C in order to harden the agar before cutting the wells.

2.1.3 Preparation of agar wells

A specially designed tube borer (Plate 2.1B) having an outside diameter of 4.5 mm was used to make 64 wells in the assay medium. A paper guide placed under the plate indicated the well positions (Plate 2.1A) and the cut pieces of agar were picked up using a sterile needle (Plate 2.1C). The well placings were randomly numbered from 1 to 16 in replicates of four.

§ *B. subtilis* spore suspension supplied by courtesy of Glaxo Laboratories, NZ Ltd.

2.1.4 Standard dilution series of penicillin

Dilutions of sodium benzyl penicillin[¶] in distilled water were freshly prepared daily and used on the plate in strengths of 5.00, 1.00, 0.1 and 0.083 iu/ml. A standard curve calculated from the zones of inhibition produced at each concentration, was plotted for each plate every time an assay was undertaken.

2.1.5 Filling the wells with test samples

Each well held a volume of 35 μ l and every specimen was assayed as four subsamples in four randomly situated wells. Thus, the technique required only a maximum of 150 μ l of the clinical specimen for the estimation. Samples were distributed using an adjustable micropipette [#] (Plate 2.1D). The assay plate contained four standard concentrations of penicillin in distilled water and twelve samples of CF containing unknown concentrations of penicillin. Each of the 16 samples was tested in quadruplicate.

The filling operation was complete within twenty minutes and the loaded assay plates were left at room temperature for one hour. This allowed the diffusion of the antibiotic into the agar before incubation at 37°C for 18-24 hours. After incubation the diameter of the inhibitory zone was measured from three directions using a vernier calliper (Plate 2.1 E), and the mean value of the three measurements was calculated.

[¶] Crystapen, Glaxo Laboratories Limited, Greenford, England

[#] Pipetman 200 (Gilson)

2.1.6 Estimation of penicillin concentration in test samples.

Standard dilutions of penicillin were transformed to logarithms to stabilize variance (Snedecor & Cochran, 1972). These transformed results of standard dilutions of penicillin were fitted by least squares analysis (Appendix 2.2). Such regression lines produced a standard curve (Appendix 2.3) from which the concentrations of penicillin in CF were estimated (Simon & Yin, 1970; Carlone & Cuffini, 1982). The calculation was carried out by fitting the above transformed data to the regression equation.

$$\hat{Y} - \bar{Y} = b (X - \bar{X})$$

where X = diameter of the inhibitory zone (mm)

$Y = \log_{10}$ penicillin concentration (iu/ml)

The errors in estimated penicillin concentrations were calculated using the formula

$$S_{\hat{Y}} = S_{y \cdot x} \sqrt{\frac{1}{n} + \frac{x^2}{\sum x^2}} \quad (\text{Snedecor \& Cochran, 1972})$$

where, $S_{\hat{Y}}$ = sample standard deviation of $\hat{Y}$ as an estimate

$S_{y \cdot x}$ = sample standard deviation from regression

n = number of observations on regression line

$x = X - \bar{X}$ (deviation from mean)

and $\sum x^2 = \sum X^2 - \frac{(\sum X)^2}{n}$

2.2 DETERMINATION OF THE DOSE-RESPONSE RELATIONSHIP AND THE HOMOGENEITY OF THE SOLVENTS IN WHICH THE STANDARD DILUTIONS WERE PREPARED

To test the suitability of alternatives to CF as a solvent, a standard dilution series of penicillin was made up in distilled water and another similar series in a CF - substitute (Appendix 2.4).

Subsequently each series was assayed under uniform experimental conditions on a single assay plate.

2.2.1 Determination of the relationship between inhibitory zones and penicillin concentrations

The regression of 'zone of inhibition' on penicillin concentration was calculated (Appendix 2.5A) as described previously (Section 2.1.6) for both series of dilutions prepared in either distilled water or CF - substitute. An analysis of variance for each regression line was calculated according to the method of Bliss (1967) to test the significance of the correlation between the penicillin dilutions and the inhibitory zone produced at each dilution (Appendix 2.5B).

2.2.2 Determination of homogeneity of regression lines derived from the assay using two different diluents

The regression lines obtained from assays on the two series (penicillin dilutions prepared either in distilled water or in CF - substitute) were compared as described by Snedecor & Cochran, 1972 (Appendix 2.5C).

2.2.3 Interpretation of statistical analysis

The statistical significance of differences was expressed according to the probability (P) of such differences having arisen by chance. In keeping with convention, a probability of 5 percent ($P < .05^*$) has been taken as 'significant'. Unless otherwise stated, the other levels of significance throughout the study are expressed as follows,

** $P < 0.01$ 'highly significant'

NS = 'non significant'

Values with common letters do not differ statistically one from another at the five percent level of probability.

RESULTS

It was observed that stock cultures of *B. subtilis* spores are very stable and suspensions stored at 4°C did not lose potency over a three year period. Pouring the agar at 60 to 70°C ensured an even distribution, and germination of spores was not impaired. Sharply defined edges and an excellent contrast between inhibitory zones and other parts of the agar, were well illustrated (Plate 2.2). The large assay plates which were described by Kavanagh (1972) proved suitable for routine use, were easily prepared and permitted 1 to 12 samples of CF to be assayed at one time under uniform conditions.

The coefficient of correlation between dose (penicillin dilution factor) and response (zone of inhibition) was close to unity on all plates tested: examples are presented in Table 2.1 in terms of the coefficient of determination (r^2), along with the regression coefficient (b).

The estimated standard error of the predicted penicillin concentrations ranged from .0400 to .1700 (Appendix 2.6). These standard errors increased in magnitude as the predicted penicillin concentration departed from the same mean (when $X - \bar{X}$ became greater), as described by Snedecor & Cochran (1972).

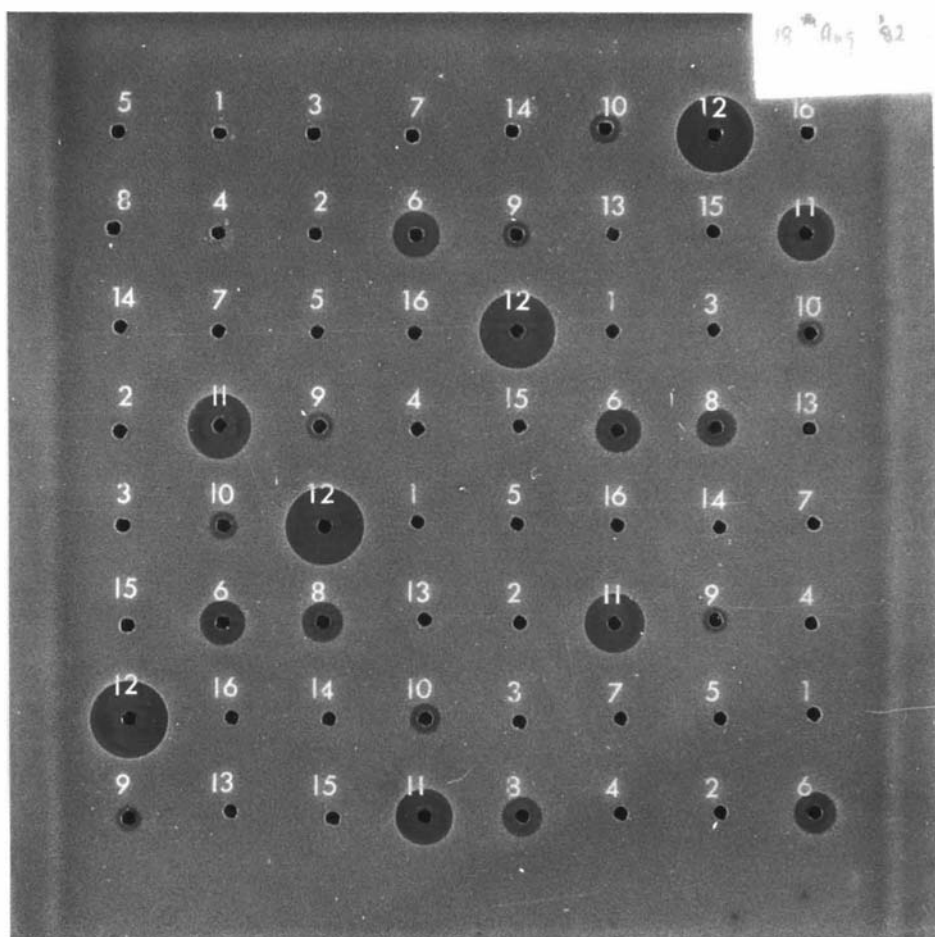


Plate 2.2 An assay plate comprising agar seeded with *Bacillus subtilis* and wells containing various concentrations of penicillin, after incubation at 37°C for 24 hours.

Well number	Test solution	Zone diameter (mm) (average of 4)	Penicillin concentration (iu/ml)
1 to 5	samples taken from different eyes before treatment	zero	zero
6	after treatment	13.55	0.5 (estimated)
7	before treatment	zero	zero
8	after treatment	12.13	0.32 (estimated)
9	} standard penicillin dilutions	8.10	0.083
10		8.89	0.10 (standards)
11		18.13	1.0
12		23.46	5.0
13 to 16	samples taken at the end of experiment	zero	undetectable

Table 2.1 Some examples of the coefficient of determination and regression coefficient derived from routine assays of penicillin standards made up in distilled water.

Plate Identity	Coefficient of determination: r^2	Regression coefficient: b
1	.9988	.1347
2	.9843	.1397
3	.9976	.1244
4	.9969	.1008
5	.9979	.1151
6	.9979	.1264
7	.9989	.1219
8	.9914	.1270
9	.9958	.1243
10	.9978	.1394

A repeatedly high coefficient determination demonstrated the existence of a close positive correlation between the variables, when either distilled water or CF - substitute was used as the penicillin diluent (Table 2.2). The highly significant F value ($P < 0.01$) rejects the null hypothesis ($\beta = 0$) and highlights the dependence of response (zone of inhibition) upon the dose (penicillin dilution factor) regardless of the diluent.

The difference between regression lines was not significant (i.e.

$F_{37}^1 = 0.0331$) when calculated from the same assay using either distilled water or CF substitute (Table 2.3).

Table 2.2. Degree of correlation between zone of inhibition and penicillin concentration when either distilled water or conjunctival sac fluid substitute was used as diluent

Source of difference					
Plate identity	diluent	n	r^2	F	Significance
1	dw	20	.9702	594	**
2	dw	20	.9526	361	**
3	dw	15	.9467	228	**
4	dw	16	.9082	9.26	**
5	dw	24	.8816	7.15	**
1	CFS	19	.9702	546	**
2	CFS	20	.9781	775	**
3	CFS	12	.9702	330	**
4	CFS	14	.9274	11.74	**
5	CFS	24	.8987	8.49	**

n = number of individual wells

dw = distilled water

CFS = conjunctival fluid substitute

r^2 = coefficient of determination

Table 2.3 Similarity of two regression lines derived from the same assay (Table 2.2) using distilled water or the substitute of conjunctival sac fluid as diluents for penicillin.

Source of difference plate identity/diluent		Regression coefficients :b	Standard error of b	F (df)	Significance	
1	dw	.233	.0096	.0331 (1:37)	NS	a
	CFS	.232	.0099			a
2	dw	.241	.0126	.6097 (1:38)	NS	b
	CFS	.226	.0082			b
3	dw	.226	.0150	6.1732 (1:25)	NS	c
	CFS	.162	.0090			c
4	dw	.247	.0210	.3846 (1:28)	NS	d
	CFS	.229	.0190			d
5	dw	.213	.0170	.0035 (1:46)	NS	e
	CFS	.211	.0150			e

dw = distilled water

CFS = substitute for conjunctival sac fluid

df = degrees of freedom

DISCUSSION

The results that confirmed the suitability of *B. subtilis* as an indicator organism for penicillin bioassay in the concentration range of interest, are in agreement with the results of Foster & Woodruff (1944). However, the particular susceptibility of *B. subtilis* towards a range of common antibiotics (Sabath, 1976) meant that the animal under study had to be protected from exposure to any other antibacterial product throughout the experimental period. The areas of error that might be encountered in the bioassay technique were identified and measures taken to minimize these variables. The repeatability and simplicity of the agar-well assay offered an accurate and convenient method to estimate the concentrations of penicillin in sequential samples of conjunctival sac fluid.

The standard penicillin dilution series prepared in either distilled water or CF - substitute, produced uniform results. However, distilled water was preferred to CF - substitute in this study as it did not demand additional preparation. For the serum assays, a standard series of penicillin concentrations was prepared in penicillin - free serum, obtained from the same animal prior to treatment.

CHAPTER 3

A SAMPLING PROCEDURE FOR BOVINE CONJUNCTIVAL SAC FLUID

INTRODUCTION

3.1 ALTERNATIVE SAMPLING TECHNIQUES

Experimental procedure

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3.1.2 Restraint

3.1.3 Paper disc method

3.1.4 Nasolachrymal duct catheter method

3.1.5 Capillary tube method

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CHAPTER 3

A SAMPLING PROCEDURE FOR BOVINE CONJUNCTIVAL SAC FLUID

INTRODUCTION

Any project designed to monitor penicillin concentrations at the site of infection by *M. bovis*, the causative organism of IBK, necessitates sequential sampling of bovine conjunctival sac fluid (CF). The duration of the experiment may need to extend for up to seven days depending on the treatment.

The conjunctival sac fluid that collects in the lower cul-de-sac comprises excess precorneal tear film. Under normal circumstances, the precorneal tear film consists of three layers, namely a superficial oily layer, a middle aqueous layer and a deep mucoid layer (Mishima, 1965) and they are derived from accessory lachrymal glands situated in the conjunctival fornices (Wolff, 1946; Botelho et al, 1969). During blinking, the lid action distributes mucous and fluid (Holly, 1973).

The constituents and the volume of CF are liable to fluctuate under different circumstances such as local irritation, pain, emotional stress and anger (Mutch, 1944; Davson & Eggleton, 1968; Best & Taylor, 1973). These fluctuations are due to the addition of secretions derived from the main lachrymal gland which responds to

any nervous stimuli (Best & Taylor, 1973). If the hope is to obtain a sample of CF representative of material at the time of collection, it is vital to select a sampling technique that will avoid any substantial stimulus so that the volume and constituents of CF remain unchanged.

Pedersen (1973) used constriction pipettes of 1000 microlitres to obtain tear samples from bovine eyes and the fluid was subsequently used for a variety of analytical techniques including bioassay. Nasolachrymal duct catheters have also been used to collect fluid from the conjunctival sac and also to estimate the tear flow-rate in cattle (Hoffmann & Spradbrow, 1978; Slatter & Edwards, 1982). For human eyes, a variety of sampling equipment has been used and this includes ultramicropipettes (Brunish, 1957), suction pipettes (Norn, 1966), microcapillary pipettes (Chrai *et al*, 1973; Broekhuysse, 1974), strips of filter paper (Josephson & Lockwood, 1964), preweighed pieces of cotton wool (Thaysen & Thorn, 1954) and surgical sponges (McClellan *et al*, 1973).

The objective of this preliminary study was to assess the suitability of three of these procedures mentioned; namely the use of paper discs, nasolachrymal duct catheters and capillary tubes.

3.1 ALTERNATIVE SAMPLING TECHNIQUES

Experimental procedure



Plate 3.1 Method of head restraint
used for the collection
of conjunctival sac fluid.

3.1.1 Animals

Cows were chosen for their quiet temperament and were selected from a dairy herd belonging to Massey University. The herd consisted of Jerseys, Friesians and their cross breeds; representative of New Zealand dairy cows.

3.1.2 Restraint

The animals were housed in individual holding pens throughout the experimental period. The selected animals were trained to tolerate a head bail and at the time of sampling, were further restrained by fastening the head halter to the headgate by means of a metal clip (Plate 3.1).

3.1.3 Paper disc method

Dry, sterile paper discs[¶] of 8.00 mm in diameter were left in the lower conjunctival sac for five minutes before removal. The saturated discs were stored in closed sterile containers at 4°C until the assay was performed: a period not exceeding eight hours.

3.1.4 Nasolachrymal duct catheter method

The nasolachrymal duct was cannulated through the lower puncta using a clear vinyl expanded catheter[§]. The nasal end of the catheter was connected to a slow suction circuit to maintain a

[¶] Biolab, Biological Laboratories Ltd., Auckland NZ

[§] Cat SVE 12, Dural Plastics & Engineering Pty, Ltd, NSW
Australia

minimum negative pressure. At the end of a day's collection, the catheter was disconnected from the circuit, coiled and then taped on to the head halter, before the animal was released into the holding pen. The suction circuit was re-established for subsequent sampling.

3.1.5 Capillary tube method

The capillary tubes[¶] were fitted with small rubber bulbs, as capillary action alone provided insufficient volume of CF.

3.1.6 Bioassay

Samples of CF obtained from untreated animals using the three methods described above, were assayed on an agar plate using the bioassay method described in Chapter 2.

Observations

Practical considerations:

The paper discs were found to absorb their maximum capacity of fluid within five minutes.

Using nasolachrymal catheters, fluid volumes of approximately 2 ml were collected from each eye over a 30 minute period. The collection of the fluid samples was not consistent because at the proximal end, the conjunctival folds acted as a valve and inhibited the flow of fluid into the opening of the catheter. In addition, a flow of lachrymal fluid over the lower eyelid was often observed.

[¶] Terumo capillary tubes, Terumo Corporation, Tokyo, Japan

Using the capillary tube method, variable amounts of fluid (150 to 250 microlitres) were obtainable from the medial canthus of each eye, usually over a period of 10 to 20 minutes.

Bioassay:

Inhibitory zones were observed on bioassay plates around the samples obtained using either paper discs or nasolachrymal catheters. However, there was no evidence of inhibitory substances in the samples collected using capillary tubes.

Discussion

Natural antibacterial substances are known to be present in some bovine and human glandular secretions. Morrison & Allen (1966) isolated lactoperoxidase from bovine lachrymal glands; an enzyme which catalyzes the oxidation reaction, producing hypothiocyanate which has been shown to inhibit the growth of *Bacillus cereus* (Bjorck et al, 1975; Zajak et al, 1981) in bovine milk.

Conjunctival sac fluid having this activity might be capable of affecting the growth of *Bacillus subtilis* in the assay system. In addition, the enzymes lysozyme (Fleming, 1922), betalysin (Ford et al, 1976) and prealbumin (Selsted & Martinez, 1982) have all been identified in human tears, although their presence, especially that of lysozyme, has not been demonstrated in bovine lachrymal secretions induced by mechanical irritation (Padgett & Hirsch, 1967). Although the identification of these inhibitory substances was beyond the scope of this study, it is likely that they are secreted from the major lachrymal gland (Davson & Eggleton, 1968).



Plate 3.2 Collection of conjunctival sac fluid from the medial canthus of the eye using a blunted capillary tube.
(N.B. Fluorescein stain has been used to improve the contrast.)

The smoothness of the blunted capillary tubes and the general care that had to be taken in collecting fluid by this method, avoided sensitive tissues (Plate 3.2) and it meant that there was no local irritation to stimulate reflex lachrymation.

In addition to the appearance of inhibitory zones around the samples that had been collected using either discs or the nasolachrymal duct catheters, both techniques caused excess stress which agitated the animals. Moreover, any long term retention of the catheter changed its physical properties and it became easily broken within the duct. All these factors collectively, made it impracticable and undesirable to use either discs or the catheter for the sequential sampling over four to seven days as required for the project.

The simplicity of obtaining fluid using the capillary tube method, meant that additional restraint of the animal was not required and such freedom minimized apprehension. The capillary tube technique was therefore selected for further investigation.

3.2 ABSENCE OF NATURAL INHIBITORY SUBSTANCES IN SAMPLES COLLECTED BY THE CAPILLARY TUBE METHOD

The CF samples collected in capillary tubes were further tested for the presence of any natural antibacterial substances that might produce erroneous results in the penicillin assay system.

Because of the extreme specificity of the enzyme penicillinase (Ogawara *et al*, 1978), it was considered that after incubation

of CF samples with penicillinase, they would not show any inhibitory activity in the bioassay system if they did not contain any antibacterial substances other than penicillin.

In carrying out a bioassay, confirmation that the active material in unknown samples is similar to that of the standards can be provided by a comparison of slopes of the dose-response regression lines derived from the two series of dilutions. If the material causing the zone of inhibition is different in the two series, it is unlikely that the dose-response lines will be parallel (Burn, Finney & Goodwin, 1950).

Experimental procedure

3.2.1 Inactivation of penicillin in CF using penicillinase

Samples of CF were collected from cows after they had been treated with penicillin. Each sample was divided into two aliquots. One of these was stored at 4°C until the assay was carried out; a period not exceeding eight hours. The other aliquot was mixed 50:1 with penicillinase[¶] (10^5 iu/ml), and incubated for three hours at 37°C. Thereafter, all samples consisting of either penicillinase-treated CF or untreated CF, were assayed for penicillin on a single plate using the assay method described in Chapter 2.

[¶] Penicillinase, Wellcome Reagents Limited, Beckenham,
England, BR3 3BS

3.2.2 Confirming the absence of natural inhibitory substances by parallelism.

A series of CF samples were obtained from treated cows at a time after penicillin administration, when it was expected that concentrations of penicillin in CF would be high and approximately in the same range, as concentrations of standard solutions of penicillin used for the standard curve. These test samples and dilutions of them were assayed on a single plate alongside a penicillin standard dilution, as described in Chapter 2. Regression lines were constructed for each of these dilution series using the method described by Snedecor & Cochran (1972). An analysis of variance for testing linearity of each regression line was carried out according to methods described in Chapter 2. The regression lines were analysed for parallelism according to the statistical procedure advocated by Snedecor & Cochran (1972) (Appendix 2.6). This enabled the activity of penicillin to be compared at all dilution levels.

Results

Inactivation with penicillinase:

The samples of untreated CF, collected after penicillin treatment of the animal, produced zones of inhibition on the assay plate. The other aliquot of the same sample which had been treated with penicillinase did not produce any inhibitory zones.

Assay for parallelism:

The dependence of the 'zone of inhibition' upon the penicillin dilution factor of the standard solutions was highly significant. There was a high degree of positive correlation between those two variables represented by the coefficient of determination (Table 3.1). The degree of correlation was equally high, both for standard dilutions of penicillin and for dilutions of CF (Table 3.1).

Regression lines derived from penicillin standards and the dilutions of CF samples assayed on a single plate, did not differ significantly in their slopes (Table 3.2).

Table 3.1 Examples of coefficient of determinations and degree of correlations derived from penicillin standards and sample dilutions (conjunctival sac fluid).

Source of difference plate/test solution	Coefficient of determination: r^2	F	df	Significance
Standard	.9894	93	1:10	**
CF/4J(R)	.9758	64	1:12	**
CF/5J(R)	.9753	84	1:14	**
Standard	.9906	50	1:10	**
CF/2T(L)	.9991	45	1:2	*
CF/3T(L)	.9900	33	1:2	*
Standard	.9842	48	1:10	**
CF/2M(R)	.9180	8	1:4	*
CF/3M(R)	.7033	13	1:4	*
CF/4M(R)	.7151	32	1:4	**
Standard	.9958	85	1:13	**
CF/2T(R)	.9380	8	1:4	*
CF/4T(R)	.9784	10	1:4	*
Standard	.9969	99	1:14	**
3J(L)	.9529	13	1:4	*

df: degrees of freedom

CF/4J(R): conjunctival sac fluid sample from the right eye
of cow number 4J

Table 3.2 Analysis for parallelism of regression lines that were derived from the dilution series of either penicillin standards or CF samples.

Source of difference plate/test solution		Regression coefficient: b	Standard error of b	F (df)	Significance of difference
Standard		.1075	.0224		
1	CF/4J(R)	.1265	.0320	0.3538	NS
	CF/5J(R)	.1158	.0254	(2:40)	
Standard		.1282	.0686		
2	CF/2T(L)	.1135	.3236	0.0067	NS
	CF/3T(L)	.1584	.3192	(2:14)	
Standard		.1348	.0569		
3	CF/2M(R)	.1436	.1253	0.0265	NS
	CF/3M(R)	.1193	.1402	(3:22)	
	CF/4M(R)	.1345	.1561		
Standard		.1209	.0080		
4	CF/2T(R)	.1291	.0393	0.0068	NS
	CF/4T(R)	.1338	.2756	(2:19)	
Standard		.1326	.0268	0.1601	NS
5	3J(L)	.1118	.0925	(1:18)	

DISCUSSION

The enzyme penicillinase completely neutralized penicillin activity present in CF samples as demonstrated by; the absence of inhibitory zones in the assay system without residual antibacterial activity. Had there been natural antibacterial substances in the samples collected, residual activity would have been expected to persist and have shown up in the assay procedure. It was concluded therefore that naturally occurring antibacterial substances were not present in CF samples collected.

The dilution series derived from CF samples collected after penicillin treatment, provided data leading to regression lines that were strictly in parallel with those of the penicillin standard and samples behaved in a similar manner on the bioassay plate at all dilution levels. If antibacterial substances other than penicillin had been present, it is unlikely that either the correlation between inhibitory zones and the dilution factor of penicillin, or parallelism, could have been sustained. The results also indicate that the antibacterial activity in CF samples was caused by the same active substance (i.e. penicillin) as that of the penicillin standards. Hence, a common regression line could be used to determine the potency of individual CF samples.

It was conclusively established from this experiment that under controlled conditions the technique of sample collection using capillary tubes did not cause either excessive lachrymation or

inhibitory substances to appear in the CF. However, in order to confirm the reproducibility of the method and its freedom from unpredictable errors, control tests were carried out during each series of experiments. Samples of CF for bioassay were always obtained before treatment and after the disappearance of penicillin from CF, to determine if any inhibitory activity was present. In addition, samples of CF that were known to contain the highest penicillin concentrations were diluted and assayed at several different levels alongside dilutions of the penicillin standard, in order to confirm parallelism.

Samples of conjunctival sac fluid obtained by using blunted capillary tubes were not contaminated with naturally occurring antibacterial substances. The technique was simple and the assay results could be accepted with confidence. The sampling procedure was adopted for all subsequent studies reported in this thesis.

CHAPTER 4

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION OF PENICILLIN AGAINST *MORAXELLA BOVIS*

INTRODUCTION

MATERIALS AND METHODS

4.1 TECHNIQUE TO DETERMINE MINIMUM INHIBITORY CONCENTRATION

4.2 CONFIRMATION OF PENICILLIN STABILITY IN HEART INFUSION BROTH

4.3 TEST ORGANISMS

4.4 CONFIRMING THE IDENTITY OF *MORAXELLA BOVIS* ISOLATES

4.5 PREPARATION OF THE INOCULUM

4.6 RANGE OF PENICILLIN CONCENTRATIONS

4.7 EXPERIMENTAL PROCEDURE

4.7.1 Positive and negative controls of the experiment

4.7.2 Determination of the Minimum Inhibitory Concentration

4.7.3 Determination of the Minimum Bactericidal Concentration

RESULTS

DISCUSSION

CHAPTER 4

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION OF PENICILLIN AGAINST *MORAXELLA BOVIS*

INTRODUCTION

The minimum inhibitory concentration (MIC) of an antibacterial substance may be defined as the lowest concentration at which the test organism does not produce any visible opacity after incubation (Cruickshank, *et al*, 1975). Thus the minimum bactericidal concentration (MBC) is the lowest concentration in which all organisms are killed. The test provides a quantitative measurement of the *in vitro* sensitivity of the micro-organism and from that value, an *in vivo* therapeutic concentration may be estimated after making allowance for some appropriate safety factor.

The objective of this study was to determine the MIC of benzyl penicillin against a number of New Zealand isolates of *M. bovis* cultured under standardized laboratory conditions.

MATERIALS AND METHODS

4.1 TECHNIQUE TO DETERMINE MINIMUM INHIBITORY CONCENTRATION

The tube dilution technique (Gould, 1960) was used in this study. This technique involves the preparation of a penicillin dilution series in Heart Infusion Broth§ (HIB). At each level of dilution a standard volume of the medium containing antibiotic was inoculated with a standard amount of *M. bovis* isolate taken

§ HIB (dehydrated), Difco Laboratories, Detroit, Michigan, U.S.A.

from an overnight culture. A reference strain of *Staphylococcus aureus*, was tested in parallel and each test was repeated three to five times.

The tube dilution method required that the antibiotic be diluted and the inoculated series incubated at 37°C for 24 hours; the temperature at which *M. bovis* shows its optimum growth characteristics (Henriksen, 1952). As the stability of penicillin dilutions in broth medium under such experimental conditions was unknown, a preliminary experiment to confirm the antibiotic stability had to be undertaken (Wick, 1964).

4.2 CONFIRMATION OF PENICILLIN STABILITY IN HEART INFUSION BROTH

A standard dilution series of penicillin in distilled water was made up to provide concentrations of 5.00, 2.5, 1.0, 0.5, 0.25, and 0.125 iu/ml. A similar series was made up using HIB as the diluent. Aliquots of both these series were assayed for penicillin on a single assay plate using the method described in Chapter 2. Further aliquots of dilutions of both series were incubated at 37°C for 24 hours and then assayed for penicillin, in parallel with a freshly prepared dilution series of penicillin in distilled water. Regression analysis (Snedecor & Cochran, 1972) was carried out to test for the degree of dependence of two variables, namely, the penicillin dilution factor in HIB and the zone of inhibition. Subsequently the regression lines constructed from an analysis of penicillin concentrations in HIB either before or after incubation, were compared (Snedecor & Cochran, 1972) for any indication of loss of activity during incubation.

4.3 TEST ORGANISMS

New Zealand and British isolates of *M. bovis* which were used in the study are detailed in Table 4.1. *Staphylococcus aureus* NCTC 6571 (the "Oxford" strain) was chosen as the reference standard; using the strain in this laboratory, penicillin had regularly shown an MIC of .038 iu/ml (Cullinane, pers. comm.).

Table 4.1 Isolates of *Moraxella bovis* and the reference strain of *Staphylococcus aureus* used in the MIC study.

Identity of organism /source	Massey Code numbers case/freeze dried	Features of isolate
National Health Institute /Wellington	830/594	N.Z. national type strain
Highden§ /Manawatu District	184/593	Isolated from a case of bilateral severe IBK
Highden§ /Manawatu District	105/589	Isolated from a case of bilateral severe IBK
Palmerston North§ /Manawatu District	804049/548	Isolated from a case showing acute IBK
Compton¶ /England	CAV2S/625	(Chandler <i>et al</i> , 1979)
<i>Staphylococcus aureus</i> "Oxford" NCTC 6571	/148	International reference strain

§ Local isolate, Faculty of Veterinary Science, Massey University

¶ Strain supplied by courtesy of Dr R.L. Chandler, Agricultural Research Council, Compton, Berkshire, England.

4.4 CONFIRMING THE IDENTITY OF *MORAXELLA BOVIS* ISOLATES

The following four tests were performed on each strain to confirm identity.

- a) Subculture on blood-agar plates
- b) Subculture on MacConkey- agar plates
- c) Culture in litmus milk
- d) Gram staining and bacterial morphology

4.5 PREPARATION OF THE INOCULUM

Freeze dried cultures of *M. bovis* were suspended in 0.5 ml HIB and streaked out on blood-agar plates to produce single colonies after incubation. Typical colonies were picked off, confirmed as *M. bovis* and similar colonies used to prepare the inoculum.

Universal bottles containing HIB were each inoculated with a single colony of a specific isolate in order to produce overnight broth cultures. A viable bacterial count was performed (Miles & Misra, 1938) on each culture and a volume of 0.1 ml used as the inoculum for each level of dilution in the subsequent MIC estimation.

4.6 RANGE OF PENICILLIN CONCENTRATIONS

The range of penicillin concentrations was extended above the highest concentration likely to be found in tissues during treatment, and downwards to a level at which the growth of the most sensitive bacteria would not be inhibited (Cruickshank et al, 1975).

Accordingly, the penicillin dilution series in HIB resulted in final concentrations of 5.0, 2.5, 1.0, 0.50, 0.25, 0.125, 0.06, 0.03, 0.015, 0.007 and 0.004 iu/ml.

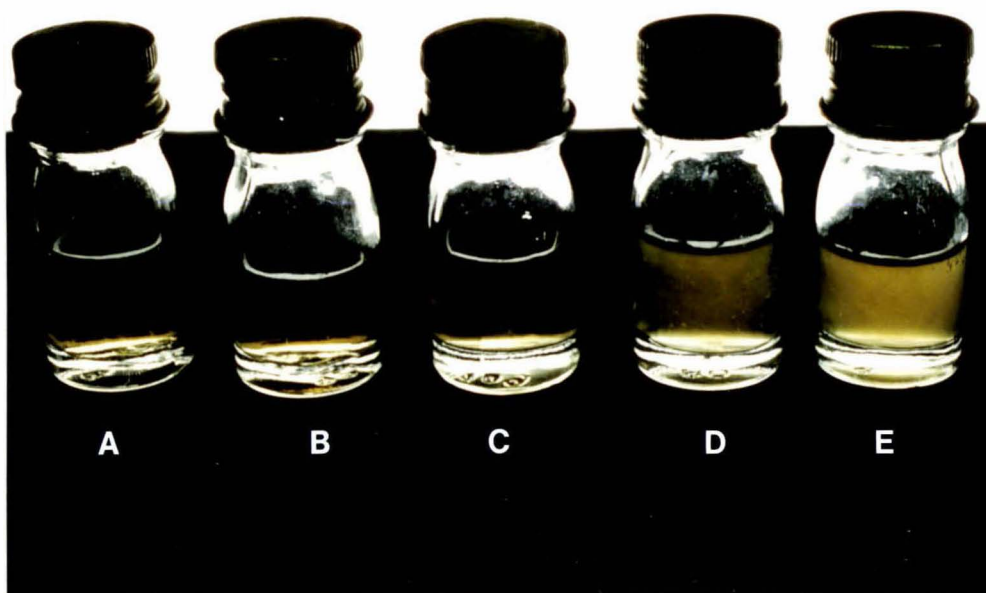


Plate 4.1 Selected tubes taken from an MIC series of penicillin against *Staphylococcus aureus* (after incubation).

- A - tube containing HIB only (negative control)
 - B & D - adjoining tubes to the end-point
 - B - no opacity
 - D - minimum opacity
 - C - tube containing the minimum amount of penicillin which prevented opacity (end-point)
 - E - tube containing HIB & *S. aureus* inoculum without penicillin (positive control)
- N.B. MIC series using *M. bovis* as the inoculum resulted in less distinctive opacity for photographic purposes.

4.7 EXPERIMENTAL PROCEDURE

A constant volume of 3 ml of each dilution was inoculated with 0.1 ml of an overnight broth-culture of a single isolate; and incubated at 37°C for 20 hours.

4.7.1 Positive and negative controls of the experiment

For each test organism, the following controls were incubated in parallel with penicillin-treated inocula described above.

- a) HIB alone (negative control)
- b) HIB + *M. bovis* (positive control)
- c) Blood agar plate + *M. bovis* (to ensure viability and purity).

4.7.2 Determination of the Minimum Inhibitory Concentration

After incubation, broth-cultures were examined visually for opacity: a comparison with both positive and negative control tubes facilitated the reading of end-points (Plate 4.1). The lowest concentration of penicillin which inhibited the development of visual opacity after overnight incubation was taken as the end-point.

4.7.3 Determination of the Minimum Bactericidal Concentration

The bactericidal end-point was taken as the tube containing the lowest concentration of penicillin; which, when all penicillin activity had been neutralized, failed to indicate viable organisms. This was determined by subculturing on blood-agar plates those broth cultures about the MIC end-point, which showed minimal or no

opacity. Residual penicillin in the broth-cultures was inactivated by incubating with the enzyme penicillinase ¶ (enzyme: broth = 1:50) for two hours at 37°C as described by Cruickshank et al (1975).

RESULTS

Stability of penicillin

The correlation between variables, namely, the penicillin dilution factor and the inhibitory zone in the assay plate, was extremely good (i.e. $r^2 = 0.98$) (Appendix 4.1) when penicillin was diluted in either distilled water or HIB. The difference between the slope of regression lines derived from penicillin dilutions in either HIB or distilled water, was not significant (Appendix 4.2). Similarly the slope of the regression lines derived from the penicillin dilutions in HIB either before or after incubation, did not differ significantly.

Identification of *Moraxella bovis*

The results of cultural, staining and biochemical tests on all isolates were consistent with the characteristics of *M. bovis* (Table 4.2) as described by Carter (1976). The viable bacterial count on overnight cultures ranged between 6×10^8 and 2×10^9 colony forming units/ml.

¶ Penicillinase, Wellcome Reagents Limited, Beckenham, England BR3 3BS

Table 4.2 Characteristics of *Moraxella bovis* strains

Isolate origin / freeze dried no.		Growth on blood-agar / MacConkey plate / plate		Litmus milk	Staining/ morphology
NHI/ Wellington	594	haemolytic colonies	- ve	+ ve	Gram negative/ typical
Highden	593	haemolytic colonies	- ve	+ ve	Gram negative/ typical
Highden	589	haemolytic colonies	- ve	+ ve	Gram negative/ typical
Palmerston North	548	haemolytic colonies	- ve	+ ve	Gram negative/ typical
Compton/ England	625	haemolytic colonies	- ve	+ ve	Gram negative/ typical

Litmus milk + ve = alkaline peptonization

Bacteriostatic and bactericidal concentrations

Apart from strain 589, all *M. bovis* isolates tested showed clearly distinguishable bacteriostatic and bactericidal end-points at a concentration of 0.03 iu/ml of penicillin and these results were reproducible (Table 4.3). The bacteriostatic end-point of *S. aureus* was reproducible at 0.038 iu/ml (Plate 4.1; tube C) but bactericidal results varied between experiments. In addition, surviving organisms of *S. aureus* were demonstrated in some of the higher concentrations of penicillin well above the MIC.

Table 4.3 Effective concentration of penicillin against
Moraxella bovis in vitro

Isolate origin / freeze dried no.		Number of experiments repeated	MIC(iu/ml)	MBC(iu/ml)
NHI/	594	3	0.03	0.03
Highden/	593	3	0.03	0.03
Highden/	589	2	0.016	0.016
Palmerston North /	548	4	0.03	0.03
Compton/	625	5	0.03	0.03
<i>Staphylococcus aureus</i>		5	0.038	inconsistent

MIC : Minimum Inhibitory Concentration

MBC : Minimum Bactericidal Concentration

DISCUSSION

As penicillin in HIB was shown to express similar activity to that observed when the antibiotic is dissolved in distilled water, it was possible to use the established bioassay technique (Chapter 2) to assay penicillin stability in HIB. The similarity between regression lines (differences in slope; not significant) derived from penicillin dilutions in HIB when assayed before and after incubation at 37°C, confirmed that the activity of penicillin did not change as a result of either dilution in the broth-medium or after incubation.

The minimum inhibitory concentration of penicillin against New Zealand isolates of *M. bovis* is taken as 0.03 iu/ml and under

the test conditions described, it was identical to that of the British strain, CAV 2S. Similar tests carried out in the U.S.A. have shown an average figure of 0.25 iu/ml (Webber *et al*, 1982). However, the relatively high sensitivity observed amongst local isolates could be attributed to the fairly recent history of IBK in New Zealand (Harris *et al*, 1980) and consequently the limited opportunity there has been for different *M. bovis* strains to be exposed to penicillin.

The mode of action of penicillin against *M. bovis* appears to be wholly bactericidal (Weinstein, 1975) and because of the dilution interval chosen for this experiment, it was not possible to demonstrate any bacteriostatic effect at a lower concentration.

Difficulties in determining the bactericidal end-point for beta-lactam antibiotics against *S. aureus* have been reported recently (Pearson *et al*, 1980; Gwynn, *et al*, 1981; Kim & Anthony, 1981; Taylor *et al*, 1983). The survival of some organisms on the walls of test tubes, including those containing quite high concentrations of penicillin, might account for the growth of these organisms on subculture.

The minimum inhibitory concentration test conditions are strictly artificial and results of such experiments cannot be extrapolated direct to the field situation. In the conjunctival sac, resident

flora, phagocytes, cellular debris and the various products of cellular metabolism are likely to increase the barrier between antibiotic and bacterium, and such metabolites may antagonize the antibiotic activity. *Moraxella bovis* lying in the crypts of the cornea (Chandler *et al*, 1983) may be protected from exposure to the drug unless it is present in high concentrations. Accordingly, a safety factor of three to five times the MIC has been generally considered as necessary before predicting a likely minimum therapeutic concentration (Weinstein, 1975; Baggot, 1980). Based on the results of this study, it was calculated that the "minimum therapeutic concentration" to ensure antibacterial activity against *M. bovis* at the site of infection would be likely to range from 0.09 to 0.15 iu/ml of penicillin. By adopting the latter threshold, it was possible in subsequent experiments to determine the "duration of therapeutic concentration" produced by a variety of penicillin treatments (Fig. 4.1).

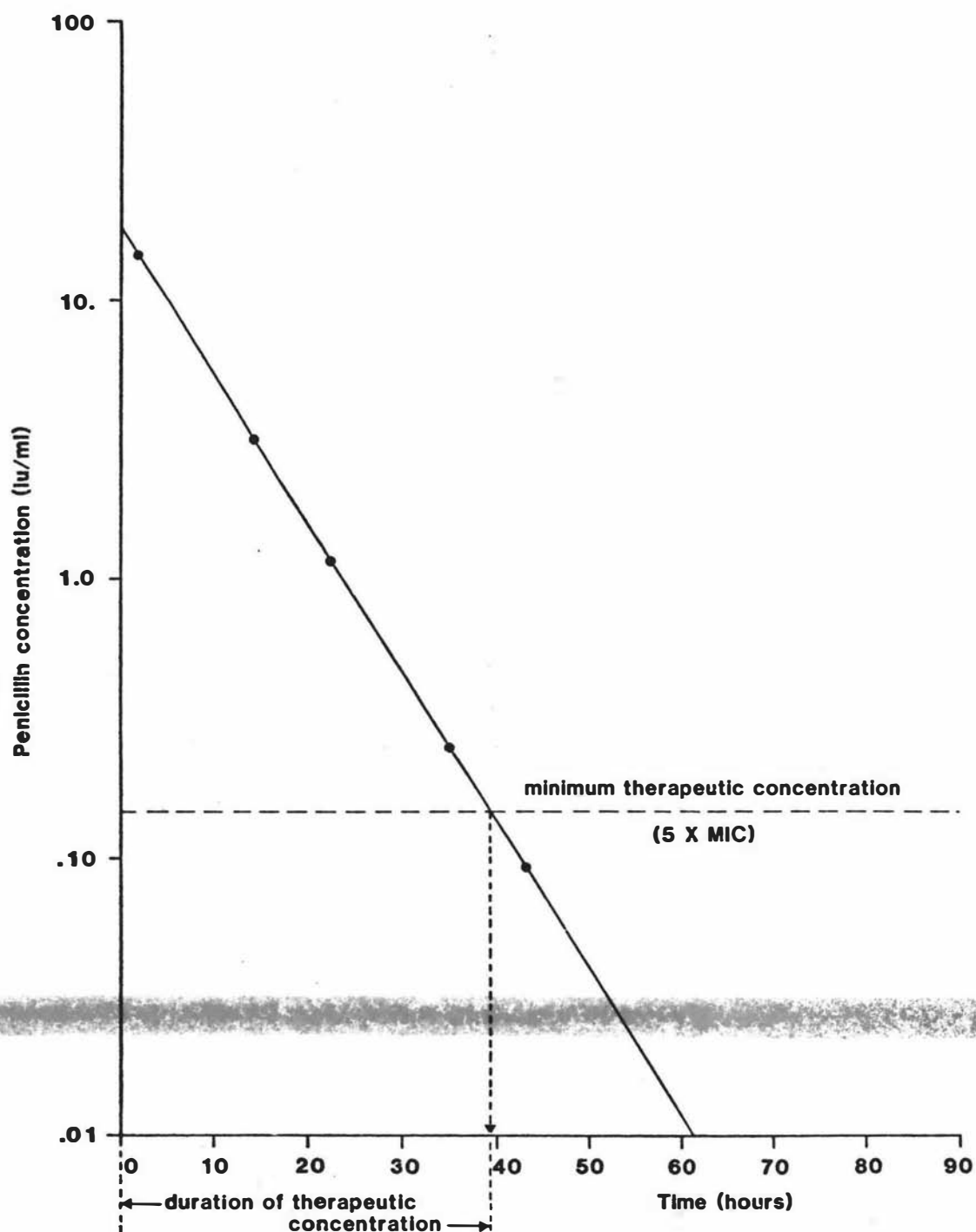


Fig. 4.1 A semilogarithmic plot depicting the exponential decay of penicillin concentration in conjunctival sac fluid and determination of the duration of therapeutic concentration.

The abbreviation S.Ct. for subcutaneous was chosen for this thesis in order that there would be no confusion with the subconjunctival route of injection. In addition the word subconjunctival was always written out in full.

CHAPTER 5

PENICILLIN CONCENTRATION IN CONJUNCTIVAL SAC FLUID FOLLOWING SYSTEMIC TREATMENT

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CHAPTER 5

PENICILLIN CONCENTRATION IN CONJUNCTIVAL SAC FLUID FOLLOWING SYSTEMIC TREATMENT

INTRODUCTION

Penicillin is one of the most widely used antibiotics in veterinary practice; a major attraction being its considerable degree of safety (Brander & Pugh, 1977). Its potential for use has been considerably extended by the production of salts and esters which possess different physical and pharmacokinetic properties.

The original salt, sodium benzyl penicillin is highly soluble in water and can be administered intravenously. It produces a very high blood concentration immediately after injection due to the instantaneous distribution within the circulatory system but this level persists for only a short time (English, 1965).

In contrast, procaine penicillin is a sparingly soluble salt of penicillin, which after extravascular administration forms a deposit at the site of the injection resulting in a slow rate of absorption (Weinstein, 1975). Formulated procaine penicillin is maintained in aqueous suspension by buffers, stabilizers and suspending agents but upon injection into the biological system, contact with tissue fluids starts the process of dissolution (Dowrick, 1980). Absorption and distribution take place over approximately 24 hours but the concentration reached in the blood is relatively low (English, 1965).

Penethamate hydriodide is the diethylaminoethyl ester of penicillin (Bleeker & Mass, 1958), having a pKa of 8.5 (Rasmussen, 1959). It is weakly alkaline in reaction, highly lipid soluble and therefore readily penetrates biological membranes (Bleeker & Mass, 1958; Edwards, 1966). The drug itself has no antibacterial activity until it hydrolyses into benzyl penicillin and diethylaminoethanol (Bleeker & Mass, 1958). Therefore, blood may contain both the basic ester and active penicillin simultaneously as a result of hydrolysis, but the ester molecules penetrate biological membranes more readily than benzyl penicillin. It has been shown that a pH difference of 0.3 between blood plasma and peripheral tissue fluids can result in concentrations of penicillin twice as high outside the circulation as that found within (Rasmussen, 1959; Pedersen, 1973). In spite of this advantage, penethamate hydriodide is an expensive product of penicillin and it is therefore desirable to use it at the lowest possible dose rate.

Pharmacokinetic analysis of drugs presents a relatively new means of interpreting the entire time course of a drug within the body. When distribution within the body is slow, a longer time may be required before equilibrium is established between the so called central (blood) and peripheral (extravascular) compartments.

For analytical purposes such a scheme represents a two-compartment open model (Notari, 1973) and the distribution of simple salts of benzyl penicillin was considered to behave according to this model (Dittert et al, 1969) after intravenous injection.

Many products of penicillin have been used in the control of eye diseases in veterinary practice but to the writer's knowledge, the relevant veterinary literature does not contain reports of the distribution of penicillin to the surface of the bovine eye, following a single dose regime. Accordingly, a series of experiments was undertaken to obtain information on the distribution of penicillin into the conjunctival sac fluid (CF). The studies involved various pharmaceutical preparations of penicillin being given by a variety of routes, as the physical characteristics of products and their site of administration are known to have an influence on both the rate of absorption and the distribution of the active ingredient in tissues (English, 1965).

MATERIALS AND METHODS

5.1 ANIMALS

Clinically normal, non lactating cows were selected from a dairy herd belonging to Massey University and were maintained indoors in individual boxes. Each experiment was performed on two animals at a time and the full series of investigations eventually involved each pair. The series of experiments was repeated using different pairs of animals in a random sequence. To ensure that any residual penicillin would have been excreted, a time interval of not less than three days was allowed between the termination of one experiment and the commencement of the next.

5.2 TREATMENTS

5.2.1 Sodium benzyl penicillin[¶], 20,000 iu/kg by intravenous

[¶] Crystavet, Glaxo Laboratories (NZ) Ltd., Palmerston North

injection (Treatment 1).

5.2.2 Procaine penicillin[¶] , 20,000 iu/kg by intramuscular injection (Treatment 2)

The total dose was administered at a single site; or when specified, the total dose was divided equally between two sites (right and left side of the animal).

5.2.3 Penethamate hydriodide[§] , 20,000 iu/kg by intramuscular injection (Treatment 3)

5.2.4 Penethamate hydriodide, 20,000 iu/kg by subcutaneous injection (Treatment 4)

5.2.5 Penethamate hydriodide, 10,000 iu/kg by intramuscular injection (Treatment 5)

5.3 SITE AND METHOD OF INJECTION

The drugs were administered by hypodermic syringe using 18 gauge needles.

- a) Intravenous injections(IV) were made into the jugular vein.
- b) Intramuscular injections (IM) were made into the cranial portion of the *biceps femoris* muscle using a 4 cm long needle.
- c) Subcutaneous injections (S.Ct.) were made into the part of the neck which was covered when the left ear was laid back flat, using a 2.5 cm long needle.

5.4 SAMPLE COLLECTION

In all experiments, both CF and blood were collected prior to the administration of the drug and periodically after treatment, until it was shown that drug concentrations had reduced to a

[¶] Mylpen, Glaxo Laboratories (NZ) Ltd., Palmerston North
[§] Leocillin, Leo Pharmaceutical Products, Ballerup, Denmark

level which could no longer be measured by the assay system.

5.4.1 Collection of blood

To facilitate frequent blood sampling over an extended period (21 to 30 days), one jugular vein of the cow was cannulated with an intravenous catheter ¶ (14 gauge x 10 cm in length) under general anaesthesia. The catheter was attached to an approximately 150 cm length of silastic tubing§ which was sutured to the skin of the neck. This tube ended in a three-way tap. The patency of the catheter and system was maintained for up to 4 weeks using heparinized # saline; a weak solution (50 iu/ml saline) being used during frequent sampling and a stronger solution (200 iu/ml saline) six hourly on nonsampling days. After general anaesthesia, the animal was rested for two to three days to ensure the elimination of residual drug.

During a single experiment, blood samples were collected at the following intervals; 0, 5, 15 and 30 minutes, 1, 2, and 3 hours and thereafter at two hourly intervals for up to 35 hours. Directly after collection blood samples were kept at room temperature for a period not exceeding two hours, thereafter centrifuged at 3000r.p.m. for ten minutes (relative centrifugal force = 1766g) and the serum taken off and stored at 4°C. The assay procedure was undertaken within eight hours.

¶ I.V. CATH, Becton, Dickinson & Company, U.S.A.

§ Medical Grade Tubing, Dow-Corning Corporation,
Medical products, Midland, Michigan, U.S.A.

Heparin B.P., Evans Medical Ltd., Speke, Liverpool

5.4.2. Collection of conjunctival sac fluid

Conjunctival sac fluid samples from both eyes were collected using sterile disposable capillary tubes as described in Chapter 3 (Section 3.1.5). During an experiment, CF samples were collected at the following intervals; 0, 15, 30 minutes, 1, 2 and 3 hours and thereafter at three hourly intervals for up to 72 hours after drug administration. The CF samples were stored at 4°C while awaiting bioassay, which was performed within eight hours of collection.

5.5 ASSAY PROCEDURE

The assay procedure adopted for both types of samples followed that described in Chapter 2. The reference standards of penicillin for the serum assay were prepared in normal serum while distilled water was used as the solvent in CF analysis.

5.6 STATISTICAL ANALYSIS

The degree of dependence of the penicillin concentration in CF (Y), on time (X), was determined using regression analysis (Chapter 2, Section 2.2). For this experiment the symbol Y in the formulae represented the $\log_{10}$ penicillin concentration, and the symbol X, time.

In addition, the standard error of the regression coefficient (S_b) and the 95% confidence limits for the slope ($b \pm tS_b$) were determined using the technique described by Snedecor & Cochran (1972). When appropriate, to enable the determination of a common regression

line for each treatment, homogeneity of regression lines was calculated by a series of F tests (Snedecor & Cochran, 1972) using the method described in Chapter 2 (Section 2.2.2).

The rate of decline of penicillin concentration in CF was compared for different treatments using the formula

$$t = \frac{b_1 - b_2}{\sqrt{Sb_1^2 + Sb_2^2}} \quad (\text{Steel \& Torrie, 1960})$$

t distribution with $(n_1 + n_2 - 4)$ degrees of freedom

where b = regression coefficient

Sb = standard error of regression coefficient.

The duration of the therapeutic concentration of penicillin in CF (Chapter 4, Fig. 4.1) was calculated for individual eyes within each treatment using the equation,

$$\hat{Y} = \bar{Y} + b (X - \bar{X}) \quad (\text{Snedecor \& Cochran, 1972}).$$

The mean duration for given treatment together with its standard error of the mean (SEM) was calculated using

$$SEM = SD/\sqrt{n}$$

$$\text{where } SD = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n}} \quad (\text{Swinscow, 1978})$$

Because the rate of decline and the duration of the therapeutic concentrations of penicillin in CF were calculated by different

means, the estimates given in the last column of Table 5.1 and the intersect of the slope of the common line depicted in Figs 5.2, 5.4, 5.5, 5.7, 5.9 and 5.11 are not strictly comparable.

5.7 DISPOSITION KINETICS OF PENICILLIN

The penicillin concentration in serum for individual animals following different forms of treatment, was calculated utilizing compartment models (Gibaldi *et al*, 1969; Ziv *et al*, 1973; Ritschel, 1976; Baggot , 1977; Notari, 1980) as follows:

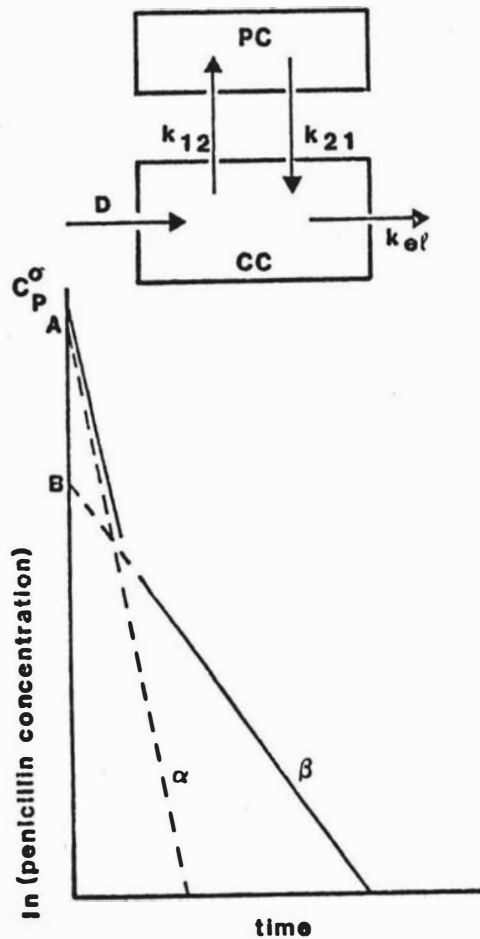
- a) The two compartment open model (Baggot, 1977) was used to evaluate sodium benzyl penicillin following intravenous administration (Treatment 1: Fig. 5.1a).
- b) The single compartment open model (Ritschel, 1976) was used to evaluate procaine penicillin and penethamate hydriodide following extravascular administration (Treatment 2, 3 & 4: Fig. 5.1b).

Details of the pharmacokinetic calculations are given in Appendix 5.1. Determination of the penicillin concentration in serum at periods after drug administration was carried out on samples from a single cow receiving any given treatment, whereas assessment of the penicillin concentration in CF was carried out on all four animals of each treatment group.

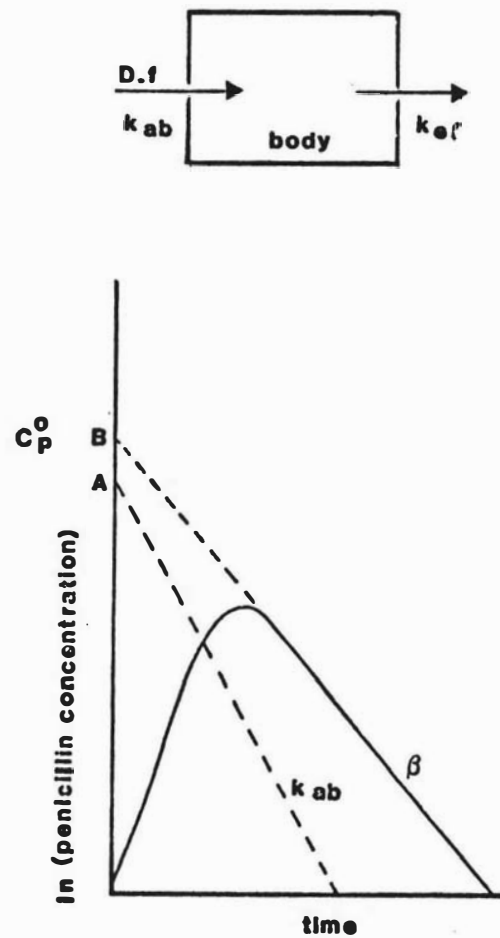
Fig. 5.1 Diagrammatic representation of pharmacokinetic analysis using compartment models.

Dose	= dose administered
f	= percent drug absorbed
k_{ab}	= absorption rate constant
CC	= central compartment
PC	= peripheral compartment
k_{12} & k_{21}	= distribution rate constants
k_{el}	= elimination rate constant
B	= intercept of back extrapolated slope of elimination phase
A	= intercept of back extrapolated slope of distribution phase or absorption phase
C_p^0	= drug concentration at zero time
β	= slope of elimination phase
α	= slope of distribution phase

a) Two compartment open model, intravenous administration



b) Single compartment open model, extravascular administration



RESULTS

Generally, an inverse relationship between penicillin concentration and time was observed for all individual experiments. A negative correlation involving highly significant F values ($P < 0.01$) was illustrated between the two variables for all experiments without exception (Appendix 5.2).

When each experiment was repeated in different animals, the observed differences in the rate of penicillin decay in CF were minimal (NS). These nonsignificant differences between repeat experiments (involving all treatments) are illustrated by the low F values (Appendix 5.3). The minimal differences observed in experiments between animals enabled the construction of a common regression line representing all replicates for each chosen treatment and these were subsequently used to compare the effects of different treatments (Treatment nos. 1-5).

Treatment 1: Intravenous injection of sodium benzyl penicillin

The intravenous injection of sodium benzyl penicillin resulted in a peak concentration in CF of 2 iu/ml on average within 15 minutes of administration (Fig. 5.2). However, the concentration in CF rapidly declined. The minimum therapeutic concentration was exceeded in the CF for only 5.5 ± 0.25 hours (Table 5.1).

In the serum, the peak concentration of penicillin was evident immediately after injection and the pattern of decline was moderately steep: it followed a similar pattern to that observed in CF. However, penicillin in serum became undetectable after five

hours (Fig. 5.3) and had a half-life of 0.7 hours (Table 5.2).

In addition, the disposition rate constants (k_{12} & k_{21}) which were 1.71 and 3.29 hour⁻¹ respectively, highlighted a rather slow tissue distribution of sodium benzyl penicillin.

Treatment 2. Intramuscular injection of procaine penicillin

After the intramuscular injection of procaine penicillin, the initial appearance of penicillin in the CF was delayed by approximately 30 minutes. Apart from one eye (40JR) a relatively low peak concentration (1.0 iu/ml) was achieved (Fig. 5.4) and maintained above the therapeutic level for 16.5 ± 1.25 hours (Table 5.1). This illustrated a very slow rate of decline for the penicillin concentration in CF. When the same total dose was divided and injected at two sites, a slightly greater persistence of the therapeutic concentration was observed in CF (Fig. 5.5 & Table 5.1), but this difference was not statistically significant.

The disposition curve for penicillin in serum illustrated two distinct phases, namely, absorption and elimination (Fig. 5.6). The peak penicillin concentration in serum was relatively low (2.0 iu/ml) but was maintained for up to 12 hours with minor fluctuations. Thereafter, the penicillin concentration in serum declined slowly and was undetectable after 29 hours. The pharmacokinetic analysis illustrated a half-life of 5.9 hours and the elimination rate constant was 0.12 hour⁻¹ (Table 5.2).

Treatment 3: Intramuscular injection of penethamate hydriodide

The intramuscular injection of penethamate hydriodide caused a

peak penicillin concentration (on average 3.0 iu/ml) in the CF within 15 minutes after administration (Fig. 5.7). Penicillin concentrations in CF persisted above the minimum therapeutic level for 57.5 ± 5.8 hours (Table 5.1).

The penicillin concentration in serum did not follow a similar pattern to that in CF. The peak concentrations in serum were achieved within two hours after injection and only after four hours (Fig. 5.8) did an excretion phase become evident. The concentration in serum showed a moderately rapid decline thereafter. Penicillin became undetectable after 14 hours. Pharmacokinetic analysis illustrated a half-life of 2.5 hours and an elimination rate constant of 0.3 hour^{-1} (Table 5.2).

Treatment 4: Subcutaneous injection of penethamate hydriodide

A subcutaneous injection of penethamate hydriodide resulted in a peak concentration in CF (average 3.0 iu/ml) immediately after administration (i.e. within 15 minutes, Fig. 5.9). The therapeutic concentration of penicillin in CF was maintained for 62.6 ± 7 hours (Table 5.1).

There were no substantial differences in the disposition of penethamate hydriodide as shown by penicillin analysis, when it was administered by either the intramuscular or subcutaneous routes. The penicillin concentration in serum did not follow a complementary pattern to that of CF. The peak concentration in serum was observed

within five hours, but thereafter it fluctuated about the same level for a further two hours (Fig. 5.10). Penicillin in serum was not detectable after 24 hours. Pharmacokinetic analysis indicated a half-life of 3.9 hours and an elimination rate constant of 0.18 hour^{-1} (Table 5.2).

Treatment 5: Intramuscular injection of penethamate hydriodide

When a low dosage (10,000 iu/kg) of penethamate hydriodide was injected intramuscularly, the peak concentration in CF was slightly lower (2.0 iu/ml) than for Treatment 3 (Fig. 5.11). The penicillin concentration in CF followed a moderately rapid decline, and the therapeutic concentration persisted only for 23.5 ± 3.8 hours (Table 5.1). A reduced dosage significantly shortened ($P < 0.01$) the period during which penicillin concentration in CF remained above the minimum therapeutic level (Table 5.1).

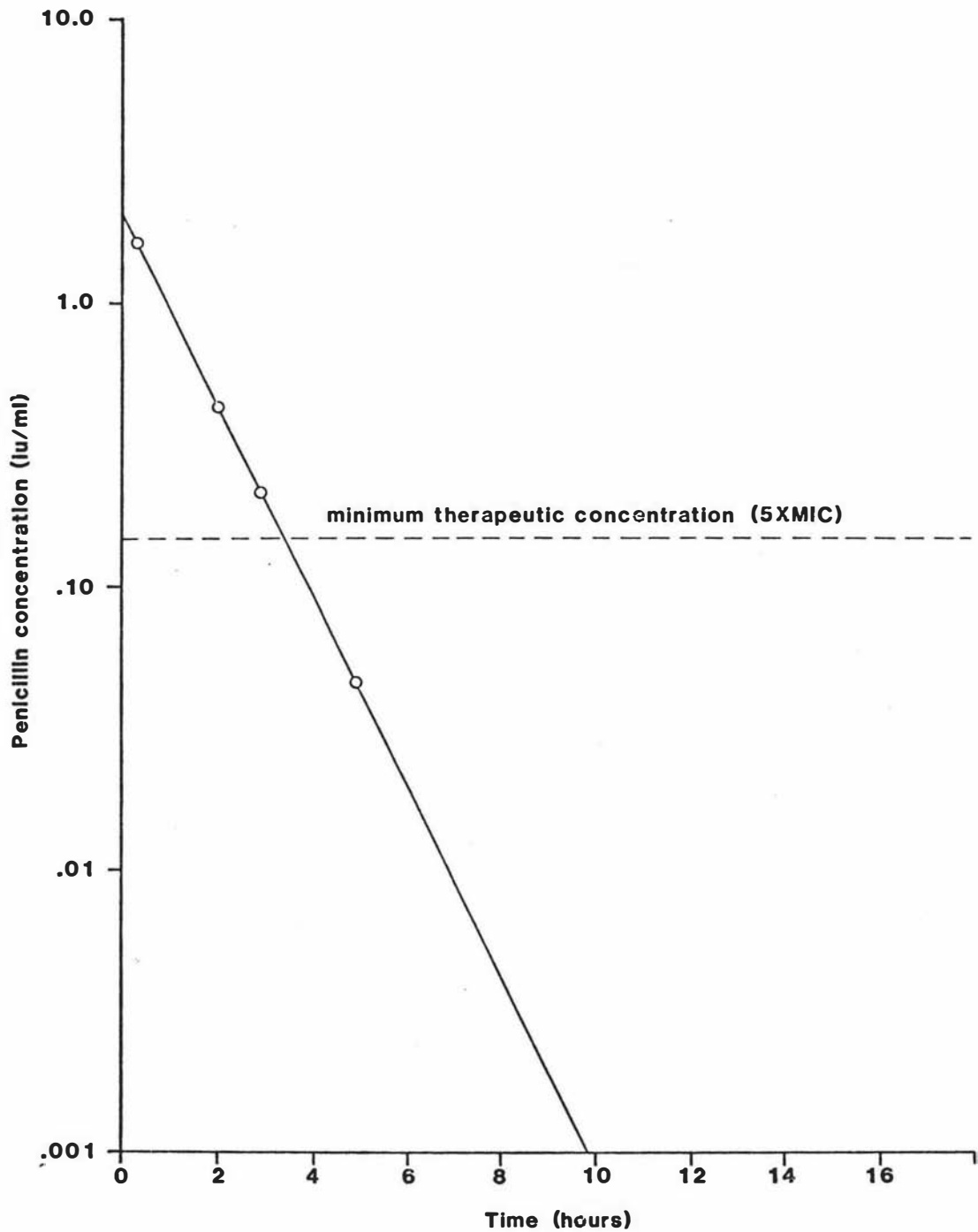


Fig. 5.2 A semilogarithmic plot of the common-regression line for penicillin in conjunctival sac fluid against time, after intravenous injection of sodium benzyl penicillin (20,000 iu/kg).

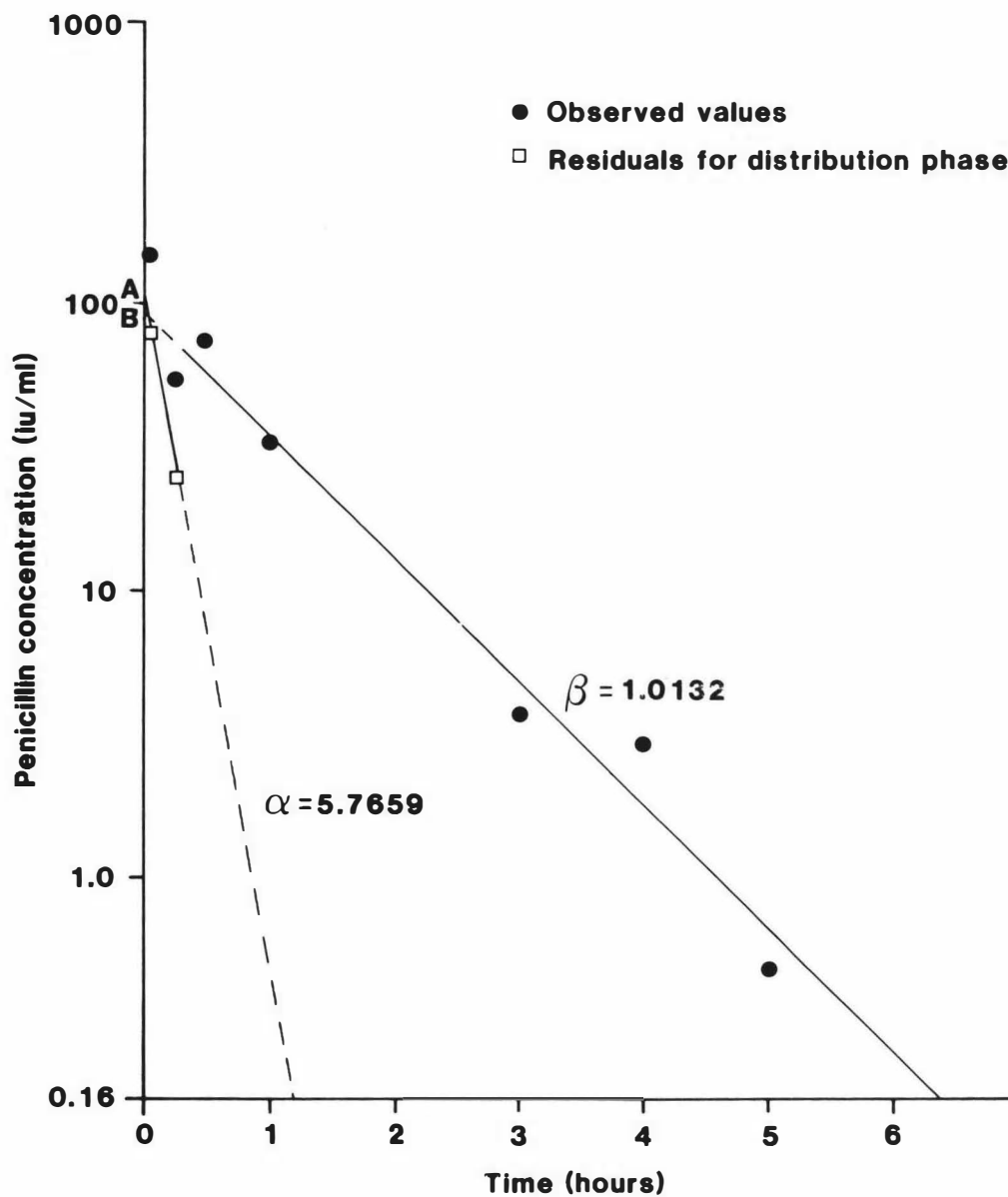


Fig. 5.3 Disposition kinetics of penicillin in serum of cow 40J, after intravenous injection of sodium benzyl penicillin (20,000 iu/kg).

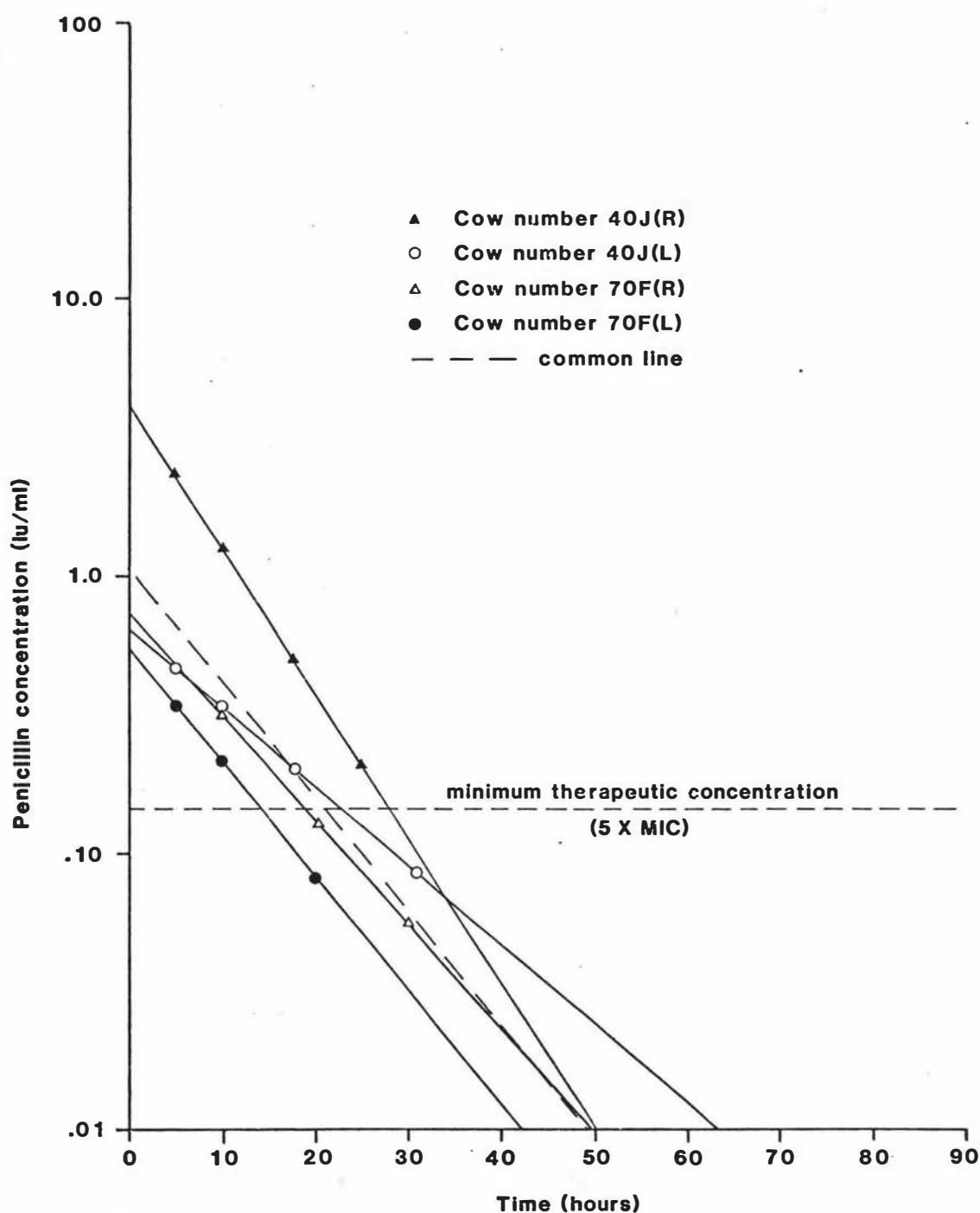


Fig. 5.4 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after intramuscular injection of procaine penicillin (20,000 iu/kg).

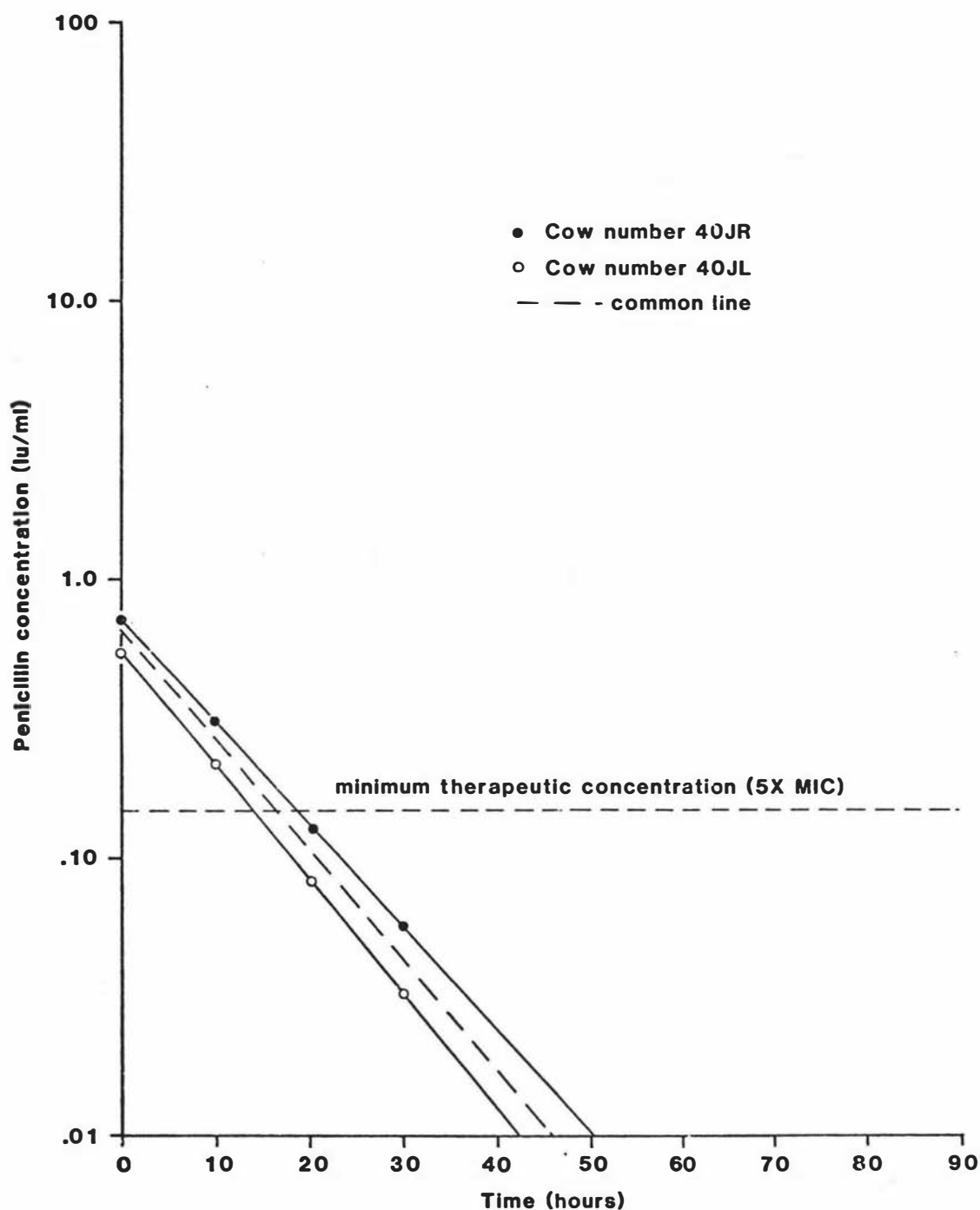


Fig. 5.5 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after intramuscular injection of procaine penicillin (divided dosage at two sites: 20,000 iu/kg).

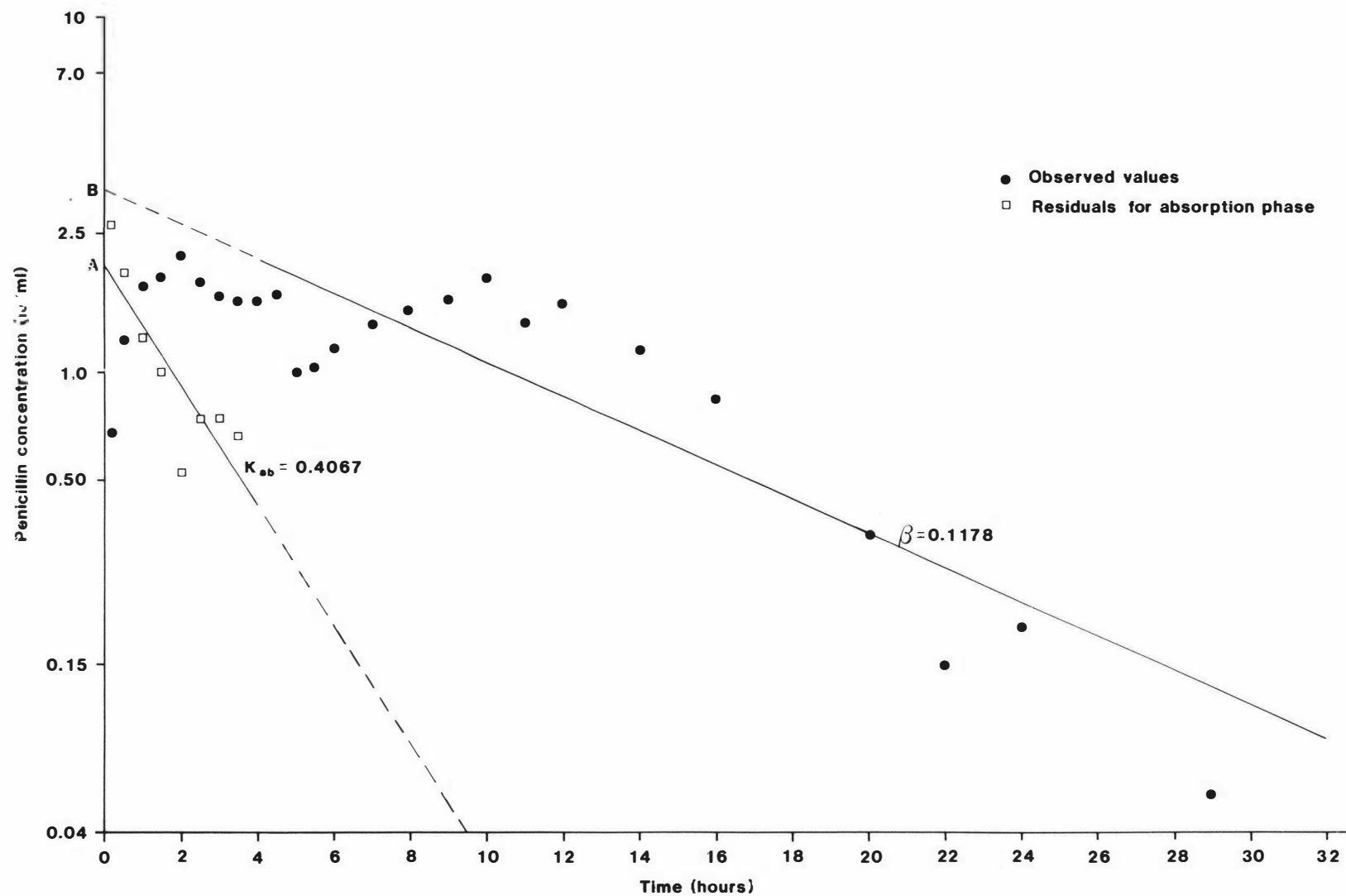


Fig. 5.6 Disposition kinetics of penicillin in serum of cow 70F after intramuscular injection of procaine penicillin (20,000 iu/kg).

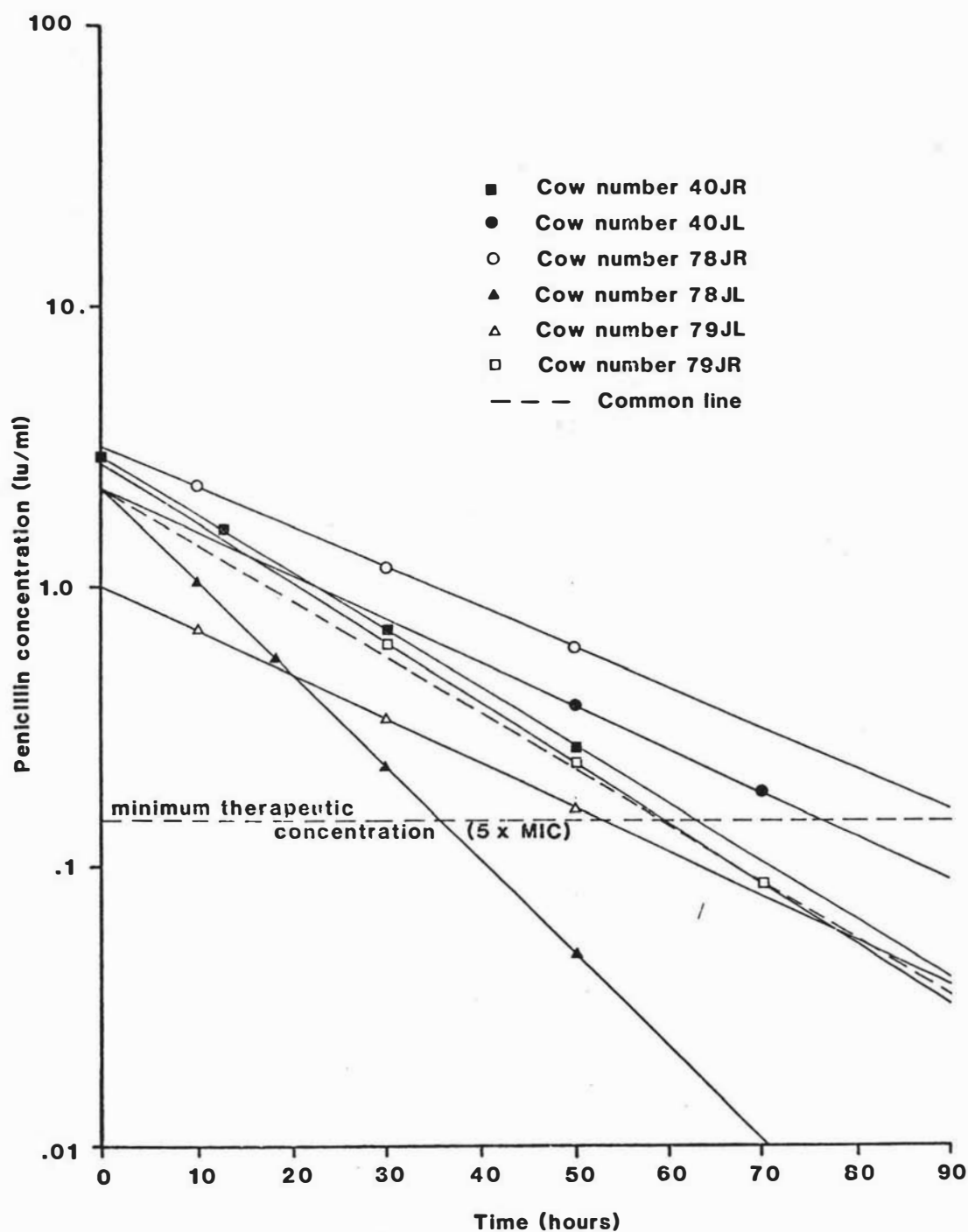


Fig. 5.7 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after intramuscular injection of penethamate hydriodide (20,000 iu/kg).

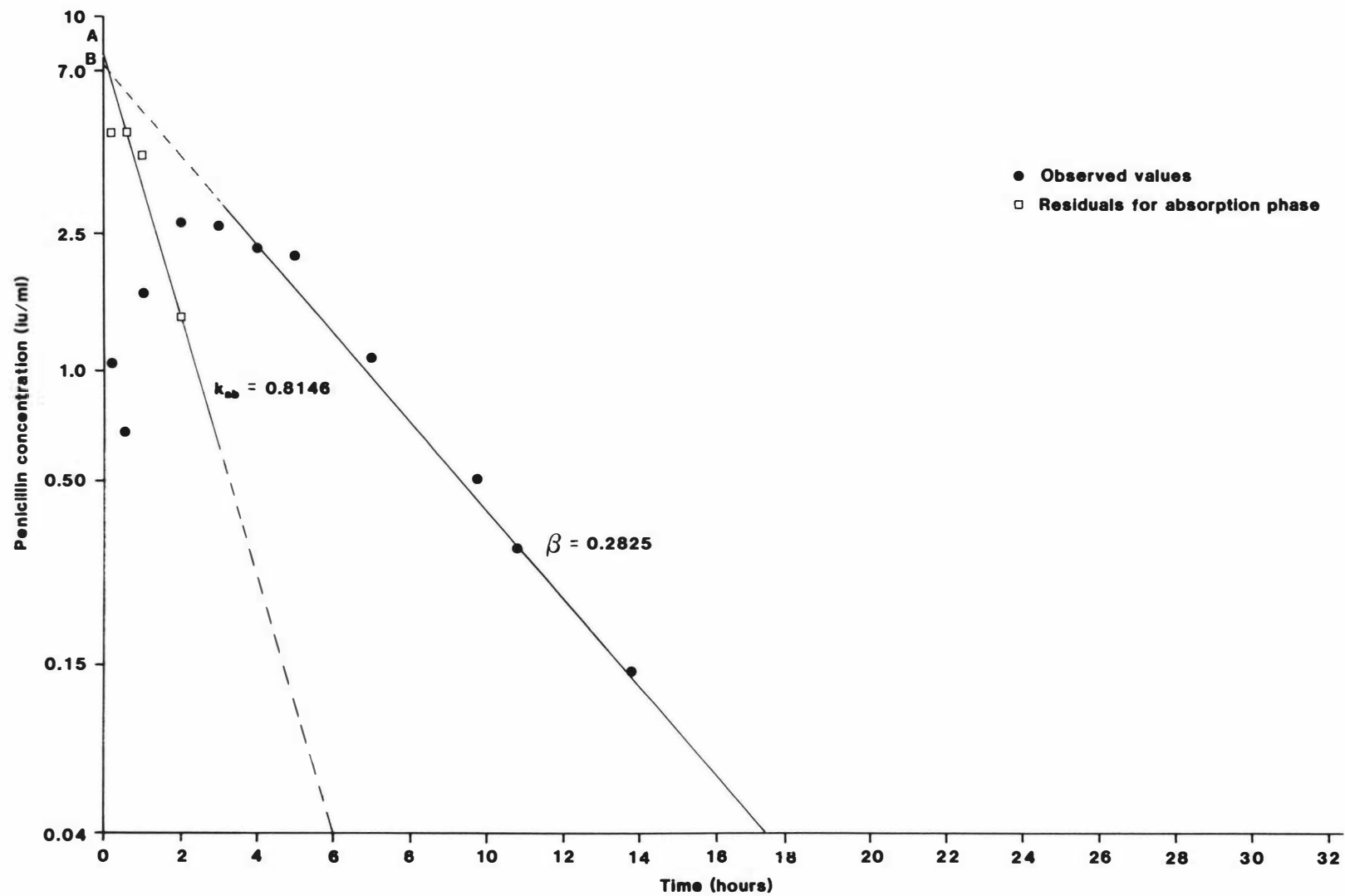


Fig. 5.8 Disposition kinetics of penicillin in serum of cow 78J after intramuscular injection of penethamate hydriodide (20,000 iu/kg).

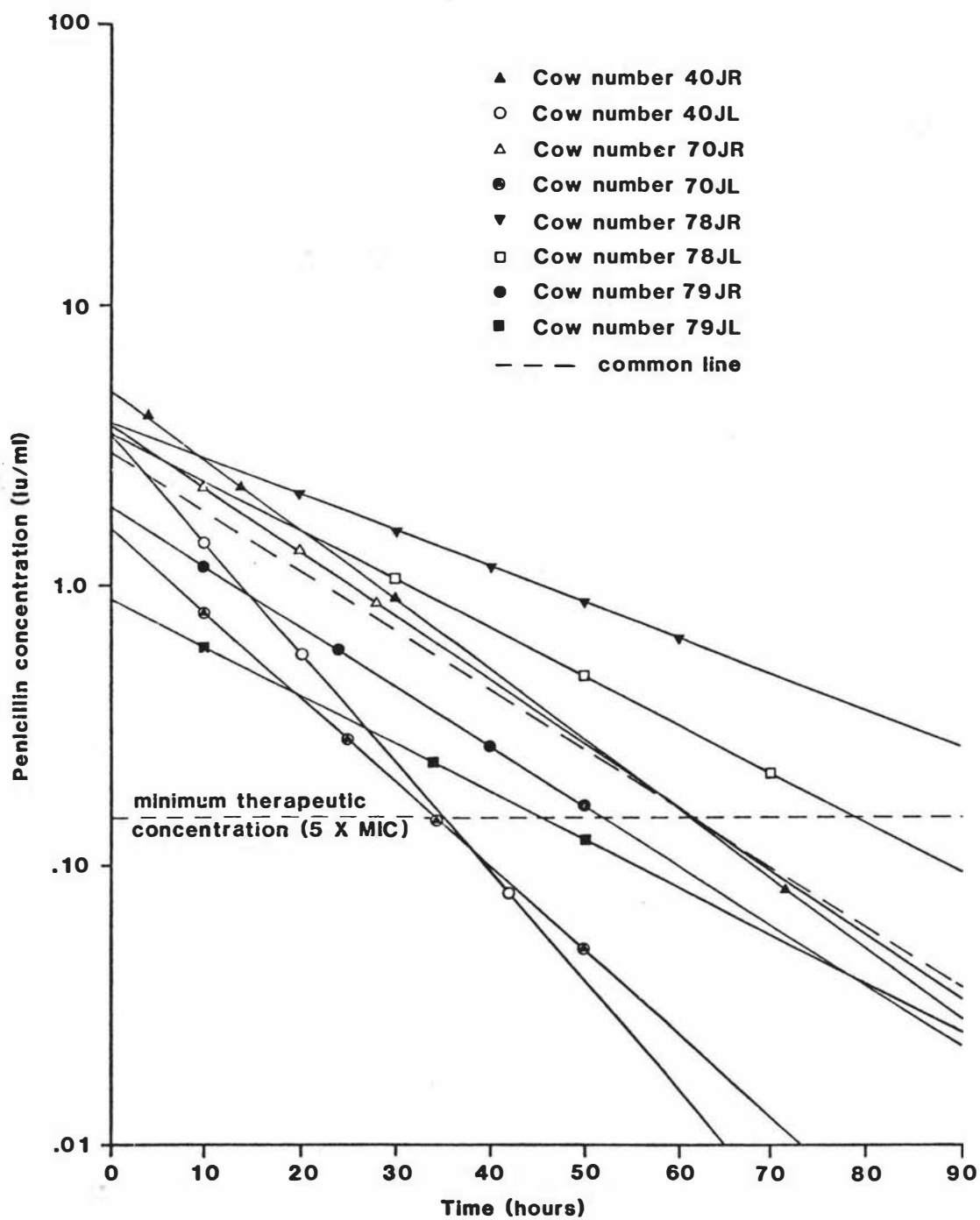


Fig. 5.9 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after subcutaneous injection of penethamate hydriodide (20,000 iu/kg).

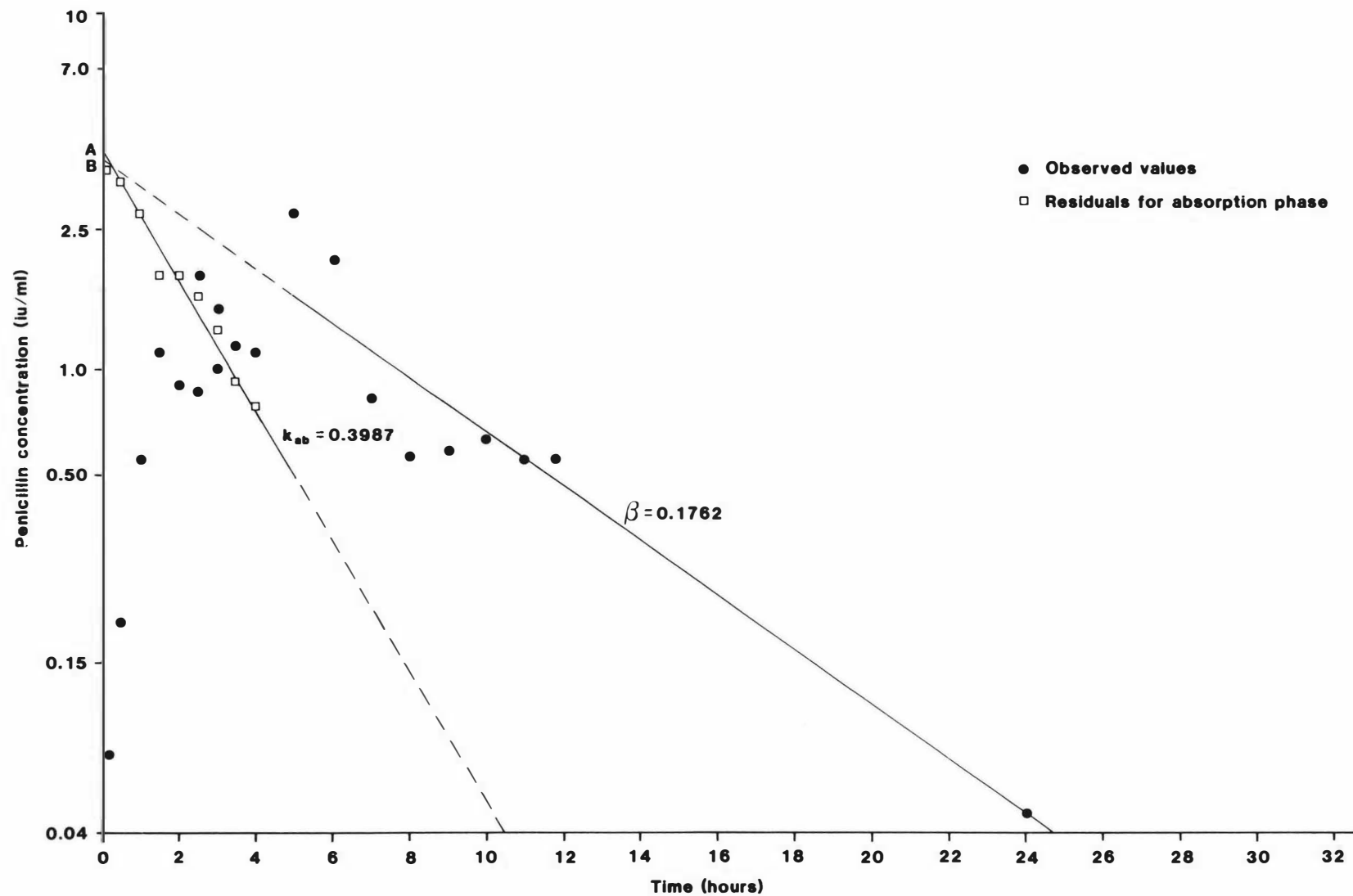


Fig. 5.10 Disposition kinetics of penicillin in serum of cow 36J after subcutaneous injection of penethamate hydriodide (20,000 iu/kg).

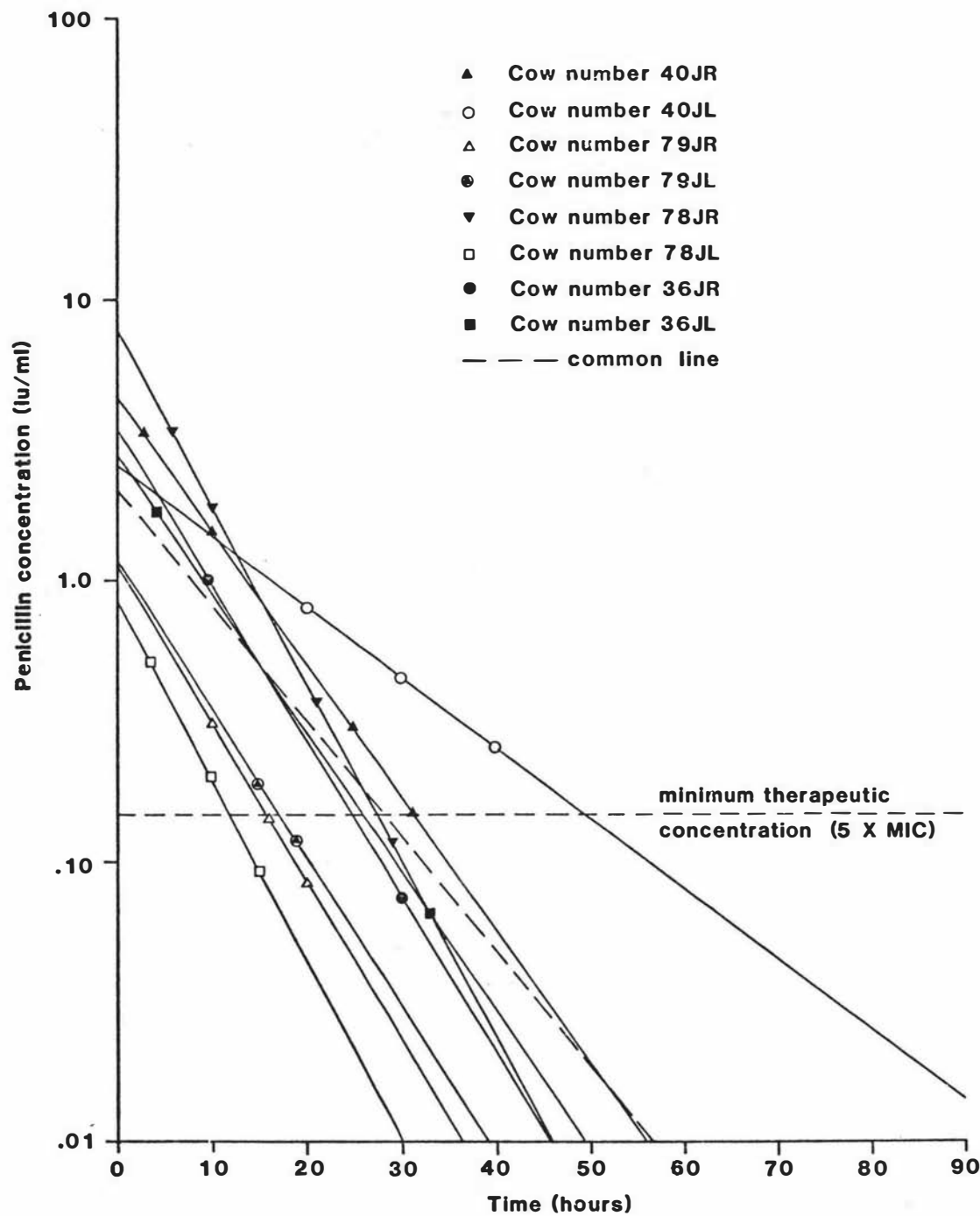


Fig. 5.11 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after intramuscular injection of penethamate hydriodide (10,000 iu/kg).

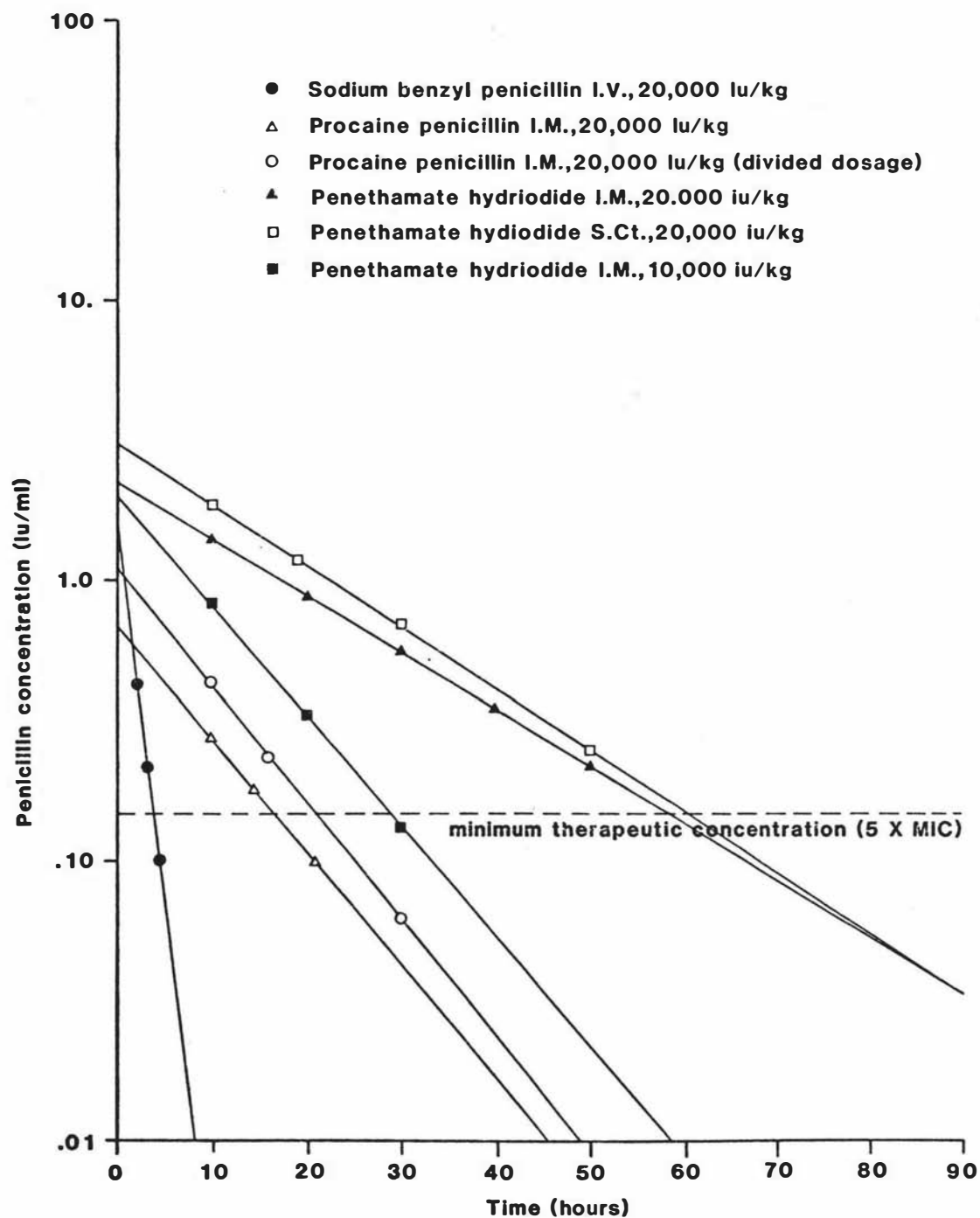


Fig. 5.12 A semilogarithmic plot of common regression lines representing the penicillin concentration in conjunctival sac fluid after systemic injections of different products of penicillin.

Table 5.1 Comparison of different systemic treatments using the common regression line of each treatment

TREATMENT number / product/dose/route		Regression coefficient of the common line ($\pm$ 95% confidence limits)	Significance of difference		Duration of therapeutic concentration: $\geq 5 \times$ MIC hours ($\pm$ SEM)
1	sodium benzyl penicillin 20,000 iu/kg, I.V.	- .3193 ($\pm$.0402)	**	a	5.5 $\pm$ 0.2544
2	procaine penicillin 20,000 iu/kg, I.M.	- .0411 ($\pm$.0111)	NS	b	16.5 $\pm$ 1.2500
2	procaine penicillin 20,000 iu/kg, I.M. (divided dosage: two sites)	- .0421 ($\pm$.0107)	NS	b	20.9 $\pm$ 3.6089
3	penethamate hydriodide 20,000 iu/kg, I.M.	- .0212 ($\pm$.0042)	NS	c	57.5 $\pm$ 5.8152
4	penethamate hydriodide 20,000 iu/kg, S.Ct.	- .0216 ($\pm$.0035)	NS	c	62.6 $\pm$ 6.9156
5	penethamate hydriodide 10,000 iu/kg, I.M.	- .0424 ($\pm$.0085)	*	d	23.5 $\pm$ 3.7915

Table 5.2 Disposition kinetics of penicillin derived from serum profiles following different systemic treatments in cattle.

Kinetic parameter	Units of measurement	Sodium benzyl penicillin (Treatment 1)	Procaine penicillin (Treatment 2)	Penethamate hydriodide (Treatment 3)	Penethamate hydriodide (Treatment 4)
dose	iu/ml	20,000	20,000	20,000	20,000
route		I.V.	I.M.	I.M.	S.Ct.
kinetic model (page 76)		two compartment intravascular	single compartment extravascular	single compartment extravascular	single compartment extravascular
chosen time when elimination phase started)		0.50 hours	4.00 hours	3.00 hours	5.00 hours
β	hour ⁻¹	1.0132	0.1178	0.2825	0.1762
B	iu/ml	97.282	3.3545	7.1958	3.8534
α	hour ⁻¹	5.7658	0.4067	0.8146	0.3987
A	iu/ml	105.52	2.10	7.8758	3.9453
C _p ^o	iu/ml	202.802	3.3543	7.1958	3.8534
t _{1/2}	hour	0.6841	5.888	2.4545	3.9338
Vd	ml/kg	172.679	n.d.	n.d.	n.d.
k _{ab}	hour ⁻¹	n.d.	0.4067	0.8146	0.3987
k ₁₂	hour ⁻¹	1.7119	n.d.	n.d.	n.d.
k ₂₁	hour ⁻¹	3.2930	n.d.	n.d.	n.d.
k _{el}	hour ⁻¹	1.7741	0.1178	0.2825	0.1762

n.d. = not done

DISCUSSION

The experiment was designed to evaluate the distribution of penicillin in CF and to determine the duration of therapeutic concentration following a single dose regime of different systemic treatments. Both the product used and the route of administration were shown to influence the penicillin concentrations in serum and subsequently in CF.

The most useful attribute of the common lines plotted in Figs 5.2, 5.4, 5.5, 5.7, 5.9, and 5.11 is that they estimate an average rate of decay for penicillin in CF derived from all individual observations with appropriate weightings (method of calculation described in Section 2.2). On the other hand, the durations of therapeutic concentrations shown in Table 5.1 are estimates derived from the unweighted means of a number of individual eyes and therefore their mean will be different from the point depicted by the common line which is a 'weighted average' line. Differences increase in magnitude as duration increases (Snedecor & Cochran, 1972).

Because sodium benzyl penicillin is a highly water soluble salt (English, 1965), it was expected that its tissue distribution would tend to be slow in keeping with the change in concentration gradient across biological membranes. In spite of this, the initial decline of penicillin concentration (α phase: Fig 5.3) can be attributed to distribution between the vascular and peripheral tissue compartments (Durr, 1976). Thereafter, the rapid serum decline is much more

likely to be attributable to the high water solubility of penicillin which facilitates its rapid renal clearance (Love *et al*, 1983). The disposition rate constants (Table 5.2) calculated for sodium benzyl penicillin highlight a rather slow tissue distribution in comparison with its relatively rapid renal excretion (English, 1965).

The rapid decline of penicillin concentration in CF can be explained both by an efficient nasolachrymal drainage system and by little if any penicillin continuing to be provided from the plasma pool, as circulating penicillin would be rapidly cleared by renal excretion.

According to the principle that increasing the dose of penicillin above 25,000 iu/kg would not proportionately increase the serum levels (English, 1965), it is most unlikely that any significant improvement in therapeutic duration in CF could be brought about by raising the dosage.

The prolonged absorption phase in the disposition curve of procaine penicillin (Fig. 5.6) may be accounted for by the gradual dissolution of the product at the site of administration (Dowrick, 1980). Such slow absorption would provide time for circulating penicillin to be excreted forthwith without accumulation in the plasma pool. Accordingly, the serum concentration never reached the threshold which would be necessary before significant distribution could take place into the CF.

The use of two sites for the administration of the total dose of procaine penicillin produced a slight improvement in the magnitude and duration of penicillin concentration in the CF (Fig. 5.12) although the effect was not significant (Table 5.1). However, this result does suggest that there was probably some improvement in absorption resulting from dividing the dose and the effect might have been due simply to an increase in the surface area between deposited drug and tissues of the host.

It was known, that if the concentration of penicillin in plasma and tissues are measured at different times following extravascular administration, the period to peak concentration in each case indicates the time required to reach equilibrium (Curry, 1977). Any group of tissues requiring the same time was considered to comprise a 'compartment'. In the case of penethamate hydriodide it was found that the time to reach a peak serum concentration never exceeded two hours and this observation together with the known high lipid solubility of penethamate hydriodide (Bleeker & Mass, 1958), permitted the use of the single compartment model for its pharmacokinetic analysis following extravascular injection.

The persistence of the penicillin concentration in CF following administration of penethamate hydriodide did not follow a similar pattern to that occurring in the serum. An early, high concentration of penicillin was observed in CF and that remained remarkably steady; above the minimum therapeutic level for a considerable period (Table 5.1). A similar extended period for penicillin concentrations

after penethamate hydriodide had been administered by different routes, was reported for bovine tears (Pedersen, 1973), aqueous and vitreous humor of rabbits (Bleeker & Mass, 1958), cerebrospinal fluid in humans (Gylfe *et al*, 1954), lung tissue of guinea-pigs (Jensen *et al*, 1951; Goslings & Hers, 1953) and bovine and ovine milk (Edwards, 1966; Ziv *et al*, 1973).

The early appearance and maintenance of a high penicillin concentration in the CF following extravascular injection could be attributed to the high lipid solubility of the undissociated penethamate hydriodide ester and its ability to transverse membranes speedily (Bleeker & Mass, 1958; Edwards, 1966). Once within the CF however, the pK_a of penethamate hydriodide, 8.3 (Rasmussen, 1959) and the pH of the CF, 7.3 (Pedersen, 1973) would have favoured dissociation to form the weakly ionized radicles of benzyl penicillin. As this dissociated product is less soluble in lipids, it is likely to be retained longer within the peripheral fluids, in this case, CF due to reduced membrane penetrability. Benzyl penicillin, was therefore observed in the CF for a long period, but in the serum a rapid decline of the penicillin concentration was observed (Fig. 5.8). This rapid decline could be attributed to the same dissociated product (benzyl penicillin) present in the blood circulation and its high water solubility, favouring rapid excretion in the urine (Love *et al*, 1983).

Neither extension nor reduction in the duration of therapeutic concentration was observed when penethamate hydriodide was administered by the subcutaneous route (Fig. 5.12). Thus, as

suggested by Marshall & Palmer (1980) in other pharmacokinetic studies, this finding adds further doubt to the established belief that blood concentrations of antibacterial products are greater following the intramuscular route of administration than the subcutaneous route.

Because of the high cost of penethamate hydriodide, half of the standard dose (10,000 iu/kg) was evaluated, but the concentration of penicillin obtained in CF and its persistence was insufficient for further consideration as a more economical treatment of IBK.

The chemotherapeutic concentrations simultaneously produced in both eyes, for all drugs tested, is a distinct advantage associated with the parenteral routes of administration. However, the study revealed that the parenteral injection of either sodium benzyl penicillin or procaine penicillin did not produce profiles in CF of adequate concentration for the treatment of superficial eye infections including IBK (Fig. 5.12). If it is decided to utilize systemic injections, penethamate hydriodide at high dosage (20,000 iu/kg) could be of some value, but treatment would be expensive.

CHAPTER 6

PENICILLIN CONCENTRATION IN CONJUNCTIVAL SAC FLUID FOLLOWING SUBCONJUNCTIVAL INJECTIONS OF PROCAINE PENICILLIN

INTRODUCTION

MATERIALS AND METHODS

6.1 ANIMALS

6.2 TREATMENTS

6.2.1 Injection through the skin (6×10^5 iu in 2 ml)

6.2.2 Injection through conjunctiva (6×10^5 iu in 2 ml)

6.2.3 Injection through conjunctiva (3×10^5 iu in 2 ml)

6.3 TECHNIQUES OF SUBCONJUNCTIVAL INJECTION

6.4 COLLECTION OF SAMPLES AND BIOASSAY

6.4.1 Conjunctival sac fluid

6.4.2 Serum samples

6.4.3 Assay procedure

6.5 STATISTICAL ANALYSIS

RESULTS

DISCUSSION

CHAPTER 6

PENICILLIN CONCENTRATION IN CONJUNCTIVAL SAC FLUID
FOLLOWING SUBCONJUNCTIVAL INJECTIONS OF PROCAINE
PENICILLIN

INTRODUCTION

Subconjunctival injections of chemotherapeutic substances have been used extensively in veterinary practice in order to increase the intraocular concentrations of poorly penetrating drugs (Wyman, 1975). In particular, the subconjunctival route of administration is widely applied in the treatment of IBK at the acute stage of the disease (Schrimsher, 1970; Fleming, 1975; Beug, 1976; Cryer, 1976; Dalton, 1976; James, 1976; Murray, 1976). In New Zealand, the use of subconjunctival injections of procaine penicillin for IBK treatment is well accepted, and it is used especially on those farms in which herds of beef cattle are brought in only occasionally from outlying paddocks for treatment. Although the clinical effects are usually satisfactory, it is still important to know from pharmacokinetic data whether or not a single subconjunctival injection of procaine penicillin could be capable of eliminating *M. bovis* infection from the treated eyes.

The subconjunctival route of administration of drugs is believed to maintain therapeutic activity in ocular tissues for an

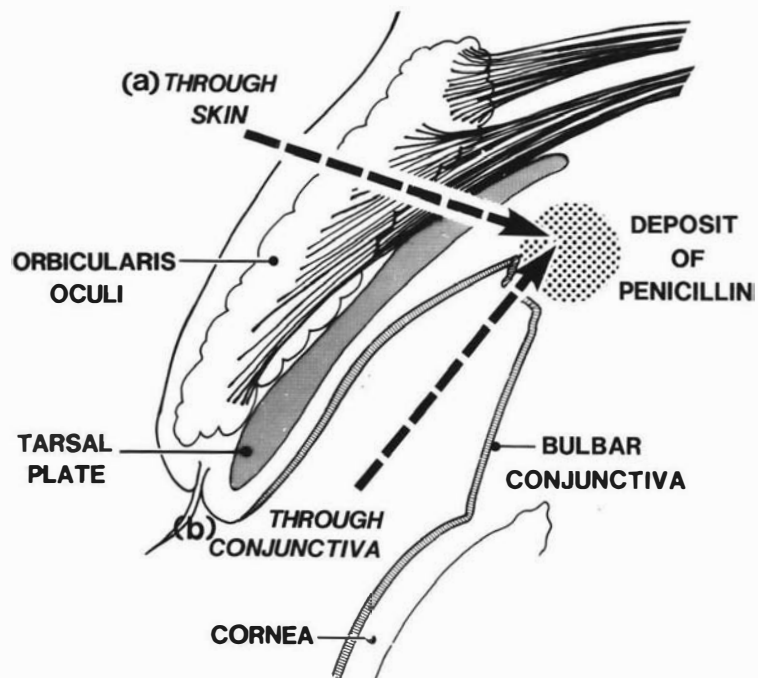


Plate 6.1 Alternative routes of subconjunctival injection.

extended period (Leopold, 1964; Gelatt, 1968; Oakley *et al*, 1976; Brightman, 1980) and yet there are no published reports on the concentrations of penicillin in CF which are obtainable after subconjunctival injection of procaine penicillin.

Amongst the few publications dealing with subconjunctival injection of antibiotics in the treatment of eye diseases, soluble penicillin has received some attention. For example Oakley *et al*, (1976) reported the presence of high concentrations of penicillin in the cornea, while Sorsby & Ungar (1946) and Andrews (1947) have dealt with the absorption of penicillin into the general circulation after subconjunctival injection.

Two routes of approach are available to reach the subconjunctival space. They are (a) injection through the skin, or

(b) injection through the palpebral conjunctiva
(Plate 6.1).

The preferred method is usually determined by custom or convenience rather than for any technical reason. Penetration of C^{14} hydrocortisone into the CF after administration by the per-conjunctival route was reported (Wine *et al*, 1964) but similar drug levels in CF were not observed when the same product was administered through the skin of the eye lid. Thus the appearance of drugs in CF after subconjunctival injection made through the conjunctiva was attributed to back diffusion of chemicals through the needle puncture (Wine *et al*, 1964).

This study was designed to investigate the magnitude and duration of penicillin occurring in CF after subconjunctival injection. It was hoped that the evidence would substantiate the use of procaine penicillin as an effective treatment method.

MATERIALS AND METHODS

6.1 ANIMALS

The animals were selected and maintained in a similar manner to that reported in Chapter 5 (Section 5.1). Each experiment was performed on two animals at a time and each pair was used in more than one experiment. A period of not less than three days after the termination of one experiment and before the commencement of another, was allowed for the elimination of any residual drug.

6.2 TREATMENTS

The following subconjunctival treatments were carried out in a random order.

6.2.1 Procaine penicillin, 6×10^5 iu (2ml) injected through the skin (Treatment 6)

6.2.2 Procaine penicillin, 6×10^5 iu (2ml) injected through the conjunctiva (Treatment 7)

6.2.3 Procaine penicillin, 3×10^5 iu (1 ml) injected through the conjunctiva (Treatment 8)

6.3 TECHNIQUES OF SUBCONJUNCTIVAL INJECTION

Procaine penicillin[¶] was administered using a hypodermic syringe and 19 gauge x 2.5 cm long needles.

6.3.1 Through the skin of the upper eye lid

The needle was inserted through the skin of the dorsal aspect of the upper eye lid close to the lateral canthus and then through the tarsal plate until the needle point had reached a position immediately beneath the palpebral conjunctiva lining the inner surface of the eye lid.

6.3.2 Through the palpebral conjunctiva

The palpebral conjunctiva of the upper-lid was everted by pressing the lower-lid over the bulb of the eye. The conjunctiva of the inner surface of the upper eye lid was pierced while the needle was being directed caudo-dorsal-medially. This was at a point one third of the distance from the lateral canthus.

6.4 COLLECTION OF SAMPLES AND BIOASSAY

6.4.1 Conjunctival sac fluid

Conjunctival sac fluid samples from both eyes were collected using sterile disposable capillary tubes as described in Chapter 3 (Section 3.1.5). Collection times and subsequent processing of CF was undertaken as described previously (Chapter 5;page 73).

[¶] Mylipen, Glaxo Laboratories (NZ) Ltd., Palmerston North

6.4.2 Serum samples

Blood samples were collected at 1 hour and 3 hours following Treatment 6, and they were processed as previously reported in section 5.4.1 (page 72).

6.4.3 Assay procedure

The general assay procedure adopted for both types of samples is described in Chapter 2 (section 2.1). As for the previous assay experiments (Chapter 3, page 54), the initial CF samples had to be free of any inhibitory substance before the assessment could be considered valid.

6.5 STATISTICAL ANALYSIS

The degree of correlation between decreasing concentrations of penicillin in CF with time, was determined using linear regression analysis (Sections 5.6 and 2.2). These individual regression lines derived from different animals were analysed for homogeneity, in order to determine the common line representing a single type of treatment. Thereafter, Treatments 6, 7 and 8 were compared using their common regression lines (Snedecor & Cochran, 1972). The time during which the penicillin concentration in CF remained above the 'therapeutic level' (Fig. 4.1) was calculated for each eye in every experiment according to the method described in Section 5.6 (page 74).

RESULTS

The penicillin concentration in CF was inversely related to time.

A high negative correlation involving significant F values ($P < 0.01$) was observed for the two variables for all individual experiments without exception (Appendix 6.1).

The rate of decline in penicillin concentration with time did not differ significantly between individual experiments using the same treatment. This included cow 40J(L) (Fig. 6.3: Appendix 6.2).

The similarity observed between eyes and animals enabled the drawing of common lines to represent each Treatment (i.e. Treatments 6, 7 and 8). Generally, a subconjunctival injection of procaine penicillin produced a relatively high concentration of penicillin in the CF immediately after injection (Figs 6.1, 6.2 and 6.3).

The subconjunctival injection given through the skin (Treatment 6) produced a significantly longer duration ($P < 0.01$) above the therapeutic concentration than administration of the same dose through the conjunctiva (Treatment 7); 67.6 ± 5 hours against 40 ± 2.7 hours respectively (Table 6.1).

When 50% of the dose in a correspondingly reduced volume (3×10^5 iu in 1 ml) was injected through the conjunctiva (Treatment 8), the duration of action (35 ± 4 hours) was less than that produced by Treatment 7 but the difference was not significant (Table 6.1).

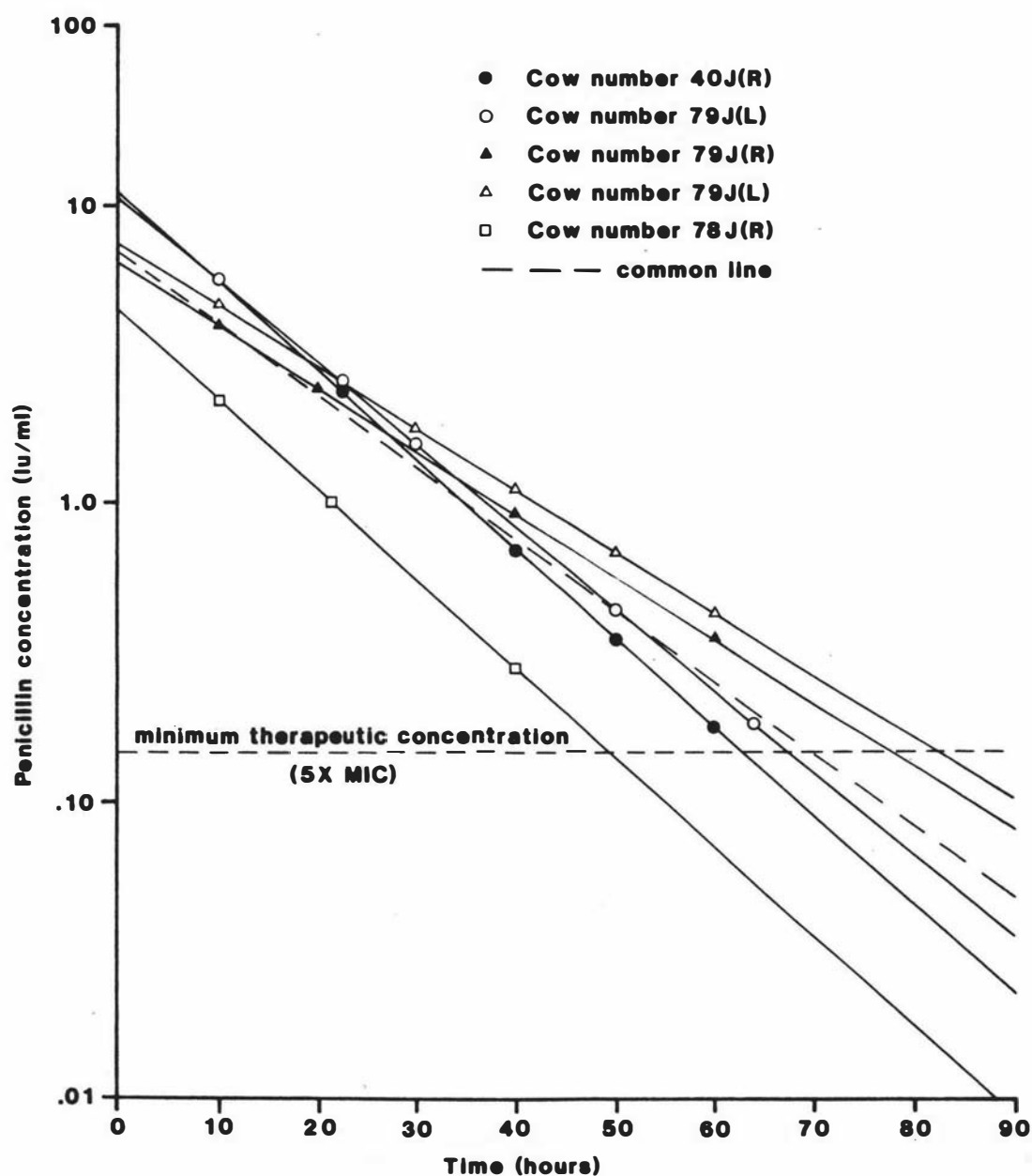


Fig. 6.1 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after subconjunctival injection of procaine penicillin through the skin of the upper lid (6×10^5 iu in 2 ml).

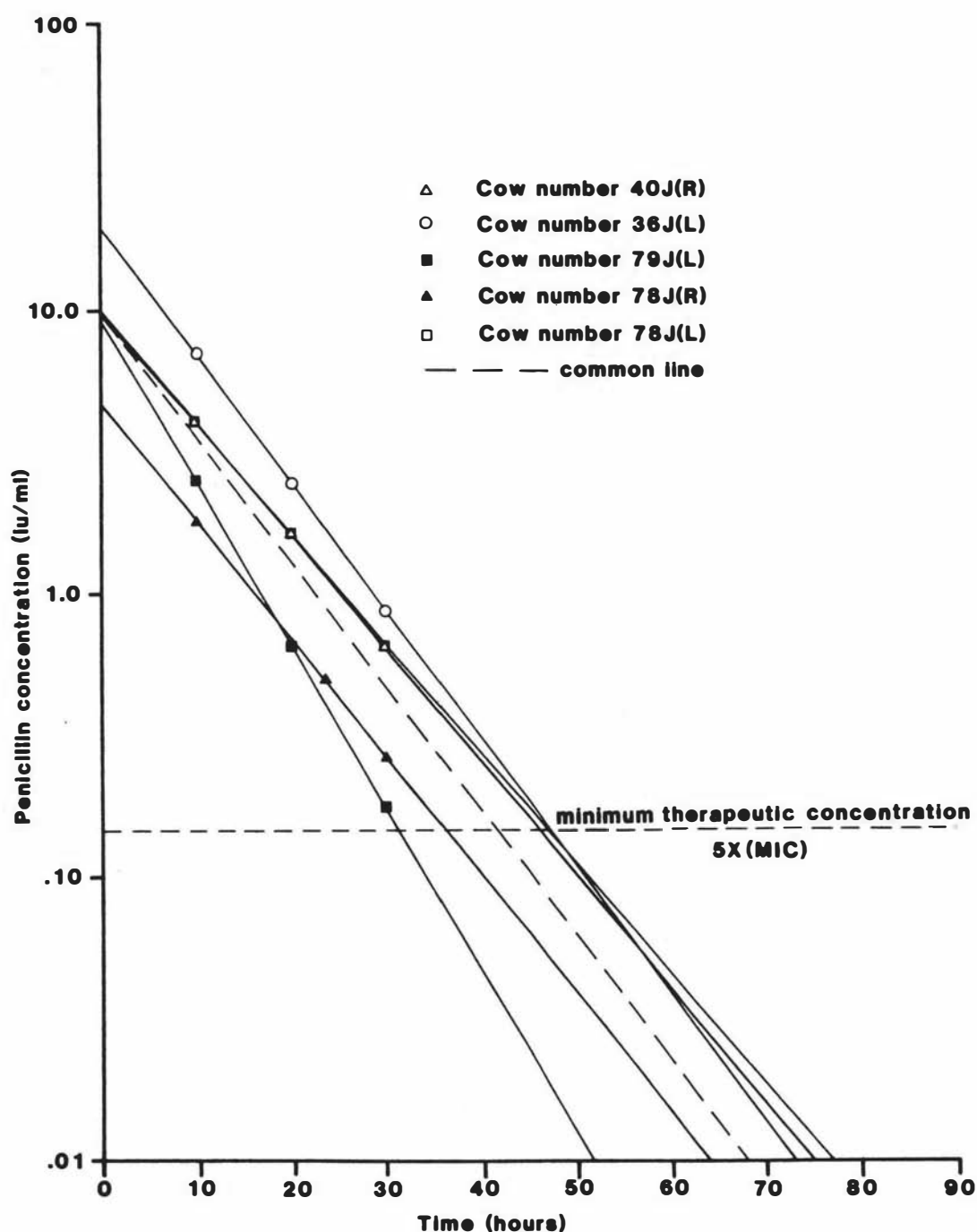


Fig. 6.2 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after subconjunctival injection of procaine penicillin through the conjunctiva (6×10^5 iu in 2 ml).

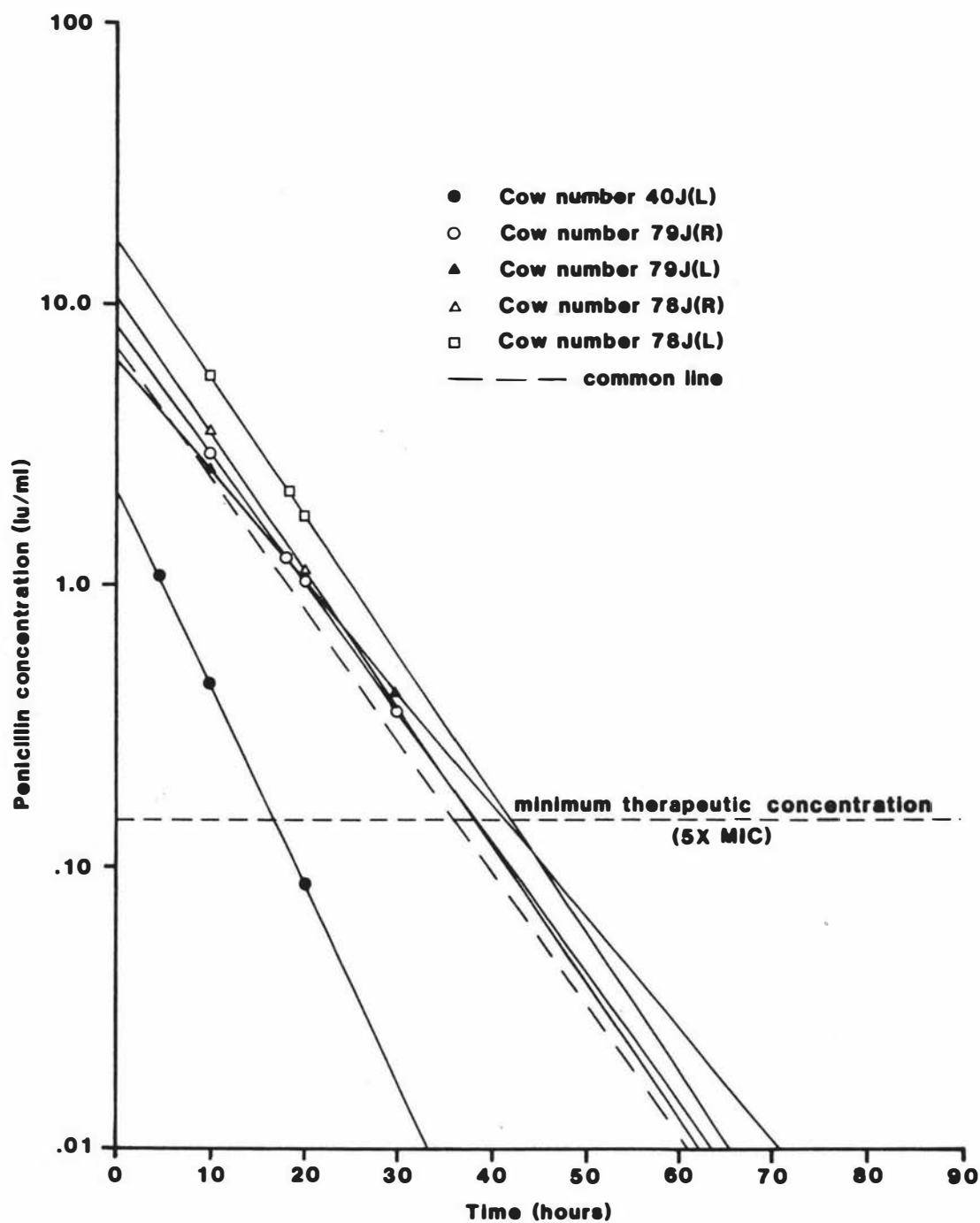


Fig. 6.3 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after subconjunctival injection of procaine penicillin through the conjunctiva (3×10^5 iu in 1 ml).

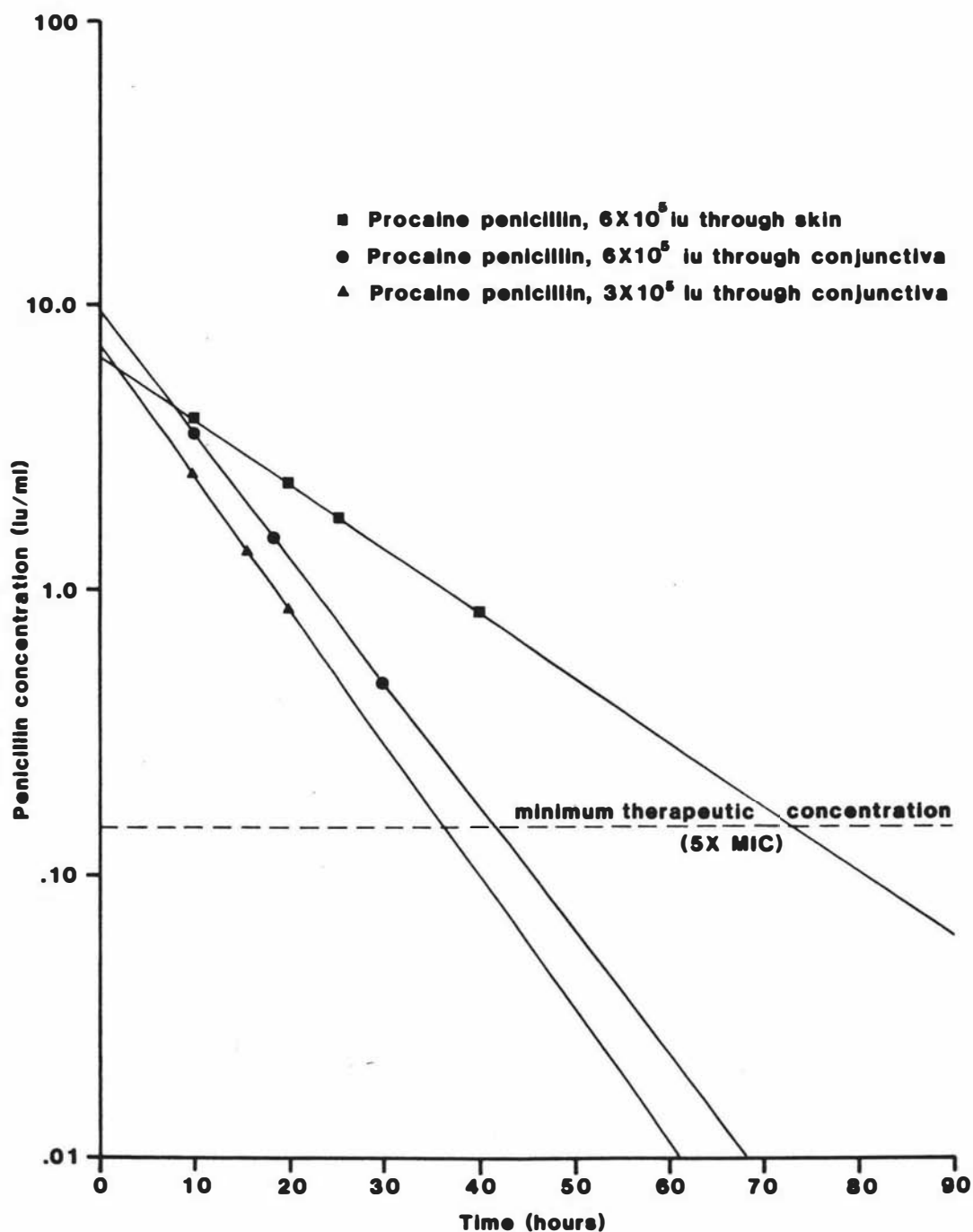


Fig. 6.4 A semilogarithmic plot of common regression lines representing the penicillin concentration in conjunctival sac fluid after subconjunctival injections of procaine penicillin

Table 6.1 Comparison of three different treatments of procaine penicillin given by the subconjunctival route

TREATMENT number / dose / route		Regression coefficient of the common line ($\pm$ 95% confidence limits)	Significance of difference		Duration of therapeutic concentration: $\geq 5 \times \text{MIC}$ hours ($\pm$ SEM)
6	6×10^5 iu through the skin	- .0245 ($\pm .004$)	**	a	67.6 $\pm$ 4.9193
7	6×10^5 iu through the conjunctiva	- .0439 ($\pm .0053$)	NS	b	40 $\pm$ 2.6743
8	3×10^5 iu through the conjunctiva	- .0481 ($\pm .0069$)	NS	b	35 $\pm$ 4.1930

The results of serum analysis indicated that penicillin is absorbed from its subconjunctival site of administration into the general circulation (Table 6.2).

Table 6.2 Penicillin concentration observed in serum after subconjunctival injection administered through the skin (Treatment 6)

Time after injection	Penicillin concentration
1 hour	3.33 iu/ml
3 hours	0.64 iu/ml

DISCUSSION

The results taken together illustrated an exponential decay of penicillin in CF (Fig. 6.4). The peak concentrations (i.e. 6-10 iu/ml) in CF observed for all treatments immediately after injection were well above the minimum therapeutic concentration of penicillin (0.15 iu/ml; Fig. 4.1). In order to have achieved these high levels, there must have been rapid diffusion of penicillin through tissues surrounding the injection site. Comparatively high concentrations (139 microgram/gram) throughout the rabbit cornea have been reported (Oakley *et al*, 1976) after a single subconjunctival injection of 'soluble' benzyl penicillin. The same authors further observed higher concentrations in the peripheral parts of the cornea than in the centre (Fig. 1.1). The peculiar distribution has been attributed to diffusion of penicillin around the corneoscleral borders (Oakley *et al*, 1976). On the other hand, in current experiments concerning the bovine eye,

simple intercellular and transcellular diffusion would account for the almost immediate appearance of penicillin in CF.

The prolonged duration of therapeutic concentration observed generally, may be attributed to the gradual and continuous release of penicillin from the sparingly soluble procaine salt (British Pharmacopoeia - Veterinary, 1977) as described in Chapter 5.

The transient uptake of penicillin from the subconjunctival site into the general circulation which was observed, is in agreement with a previous report by Sorsby & Ungar (1946).

A significantly shorter ($P < 0.01$) duration above the therapeutic concentration was observed when the drug was injected through the conjunctiva, rather than through the skin. Some injected material was observed issuing immediately from the needle track and this feature could account for the shorter period of activity as previously observed by Wine *et al* (1964). However, both techniques of drug administration into the subconjunctival site, produced sufficient concentrations of penicillin in CF, for an adequate length of time. In such circumstances, an organism as sensitive as *M. bovis* should be adequately controlled.

The reduction of the dose (Treatment 8) did not significantly change the initial concentration in CF nor the rate of penicillin

decay and therefore the duration of therapeutic concentration was not materially influenced.

The current study therefore supports the widespread use of penicillin by subconjunctival injection in the treatment of IBK. If it was felt necessary, the therapeutic concentration of penicillin produced by a single dose could be further extended by a second injection administered 2 - 3 days later.

Penicillin profiles in CF obtained from these studies were derived from clinically normal animals. It would be necessary to conduct trials using animals affected with IBK, if claims for therapeutic effectiveness were to be investigated.

CHAPTER 7

PENICILLIN PROFILES IN CONJUNCTIVAL SAC FLUID AFTER TOPICAL APPLICATION OF DIFFERENT SALTS OF PENICILLIN

INTRODUCTION

MATERIALS AND METHODS

7.1 ANIMALS

7.2 TREATMENTS

7.2.1 Sodium benzyl penicillin in distilled water

7.2.2 Sodium benzyl penicillin in ointment base

7.2.3 Procaine penicillin in ointment base

7.2.4 Benethamine penicillin in ointment base

7.3 METHODS OF INSTILLATION

7.3.1 Aqueous preparation

7.3.2 Ointments

7.4 SAMPLE COLLECTION AND BIOASSAY

7.5 SUITABILITY OF TOPICAL TREATMENTS

7.6 STATISTICAL ANALYSIS

7.7 DURATION OF THERAPEUTIC CONCENTRATION

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DISCUSSION

CHAPTER 7

PENICILLIN PROFILES IN CONJUNCTIVAL SAC FLUID AFTER
TOPICAL APPLICATION OF DIFFERENT SALTS OF PENICILLIN

INTRODUCTION

The cornea, a major component of the eye, is covered by a structured layer of fluid approximately 7-9 micrometers thick, which is referred to as the precorneal tear film (Ehlers, 1965b; Mishima *et al*, 1966). Under normal circumstances, fluid making up the precorneal tear film is derived from various local glands of the conjunctiva (Wolff, 1946), and excess material is collected in the lower cul-de-sac of the eye, where it is known as conjunctival sac fluid (CF). The conjunctival sac together with the cornea, is covered by a single continuous sheet of epithelium, which is constantly kept moist by the same tear film and in this way supplies the metabolic needs of the avascular cornea (McEwen, 1962). The fluid collected in the conjunctival sac is dispersed by the lid action during blinking (Holly, 1973) and also by the movements of the folds of the conjunctival mucous membrane; a structure which has been described by Ehlers (1965a) as "perfectly moveable and yielding".

Studies dealing with the dynamics of superficial fluids on rabbit eyes have shown that the loss of instilled solution is related to the volume of solution instilled and the rate of disappearance follows first order kinetics (Chrai *et al*, 1973). The rapid decay

has been attributed partly to nasolachrymal drainage of the instilled solution (Chrai *et al*, 1973; Wyman, 1975) and partly to absorption through the cornea and conjunctiva (Maurice, 1962). However, when drugs in solution are instilled, the viscosity and the nature of the vehicle in which the drug is dissolved is considered to have a major effect in determining the rate of drug decay (Linn & Jones, 1968; Havener, 1974; Wilson *et al*, 1983).

The purpose of the experiments that are described in this chapter, was to determine the penicillin profiles in CF resulting from the topical application of certain penicillin preparations. It was hoped that such information would be useful for advice on topically applied medication.

Therapeutic ophthalmic solutions are reported to be acceptable if they have a tonicity equivalent to 0.9% saline (Sorsby & Ungar, 1946; Martin & Mims, 1950). Therefore different salts of penicillin, having different degrees of solubility, were used at the same tonicity and at the same dose rate in either an aqueous or a bland ointment base. The topical treatments were also evaluated for their freedom from causing irritation on the normal bovine eye.

MATERIALS AND METHODS

7.1 ANIMALS

The cows were selected and maintained in a similar manner to that

described in Chapter 5 (Section 5.1). Experiments were performed on two animals at a time. One eye received the penicillin treatment while the contralateral eye received the placebo treatment. Each pair of animals was used in more than one experiment after allowing a period of not less than three days between the termination of one experiment and the commencement of the next. The treatments were repeated in different animals chosen in a random order.

7.2 TREATMENTS

Penicillin salts were incorporated in either distilled water or a bland ointment base consisting of liquid paraffin (5%) and soft white paraffin (95%) (Swinyard, 1975). Each preparation was formulated so that the mass of material administered into each eye contained 5,000 iu of penicillin in either 0.1 ml of aqueous solution or 0.3 gram of ointment.

Treatments comprised

7.2.1 Sodium benzyl penicillin[¶] 5,000 iu in 0.1 ml distilled water (Treatment 9)

7.2.2 Sodium benzyl penicillin 5,000 iu in 0.3 gram ointment base[§] (Treatment 10)

7.2.3 Procaine penicillin 5,000 iu in 0.3 gram ointment base[§] (Treatment 11)

7.2.4 Benethamine penicillin 5,000 iu in 0.3 gram ointment base [§] (Treatment 12)

[¶] Crystapen, Glaxo Laboratories Limited, Greenford, England

[§] Supplied by courtesy of Glaxo Laboratories, N.Z. Ltd. Palmerston North.

The placebo treatment consisted of either 0.9% saline or the bland ointment base .

Each topical treatment was repeated in five animals chosen in a random order.

7.3 METHODS OF INSTILLATION

7.3.1 Aqueous preparation

Two drops (0.1 ml) of sodium benzyl penicillin solution in distilled water were instilled into the lower conjunctival sac using a dropper pipette. The same volume of 0.9% sterile saline was instilled into the contralateral eye.

7.3.2 Ointments

Approximately 0.3 gram containing a total of 5,000 iu benzyl penicillin was delivered directly into the lower cul-de-sac.

The amount of ointment that needed to be introduced in order to deliver 5,000 iu penicillin was estimated beforehand and confirmed for each experiment by weighing the tubes before and after treatment.

The same amount of ointment base was delivered into the conjunctival sac of the contralateral eye (placebo treatment).

Care was taken to avoid any overflow or mechanical irritation of the eye. The volume applied was based on the studies of Mishima *et al* (1966) who estimated the average normal lachrymal volume of humans to be 7 microlitres and they suggested that the human conjunctival sac could accommodate approximately 30 microlitres without overflowing.

7.4 SAMPLE COLLECTION AND BIOASSAY

Samples of CF were collected from both eyes, one directly after the other, using sterile disposable capillary tubes as described in Chapter 3. Collection times and subsequent processing of CF was undertaken as described previously (Chapter 5; page 73).

7.5 SUITABILITY OF TOPICAL TREATMENTS

Before and after instillation of treatments, the eyes of all animals were studied for signs of local irritation. They were visually examined for clinical signs such as lachrymation, vascularization, blepharospasm or corneal opacity.

During the course of every sampling schedule, randomly selected subsamples were centrifuged in a Cytospin centrifuge[¶] for five minutes (relative centrifugal force = 197g). The cell button obtained was stained using MacNeal's technique (1922; cit. Gurr, 1960) in order to determine any changes in the number or the ratio of different cell types.

7.6 STATISTICAL ANALYSIS

Changes in the penicillin concentration in CF with time were determined using linear regression analysis in the manner described in Sections 2.2 (Appendix 2.6) and 5.6. These individual regression lines were analysed for homogeneity^e, in order to determine the common line representing each treatment. Thereafter treatments were compared using their common regression lines (Snedecor & Cochran, 1972).

[¶] Shandon SCA 0030, Shandon Southern Products Ltd.
93-96 Chadwick Road, England WA7 IPR.

7.7 DURATION OF THERAPEUTIC CONCENTRATION

The duration of therapeutic concentration of penicillin in CF was calculated for each treatment according to the method described in Chapter 5 (Page 74).

RESULTS

The eye drops were retained within the conjunctival sac and overflow was not observed. The ointment melted quickly and spread in between the folds of the conjunctiva and into the precorneal tear film.

The topical treatments used in this study did not produce clinical signs of local irritation except for a slight increase in tear flow which disappeared almost immediately. No changes were observed in the total number of cells or in differential cell counts of CF, even after repeated treatments.

Generally, an inverse relationship between penicillin concentration and time was observed for all individual experiments. A negative correlation involving highly significant F values ($P < 0.01$) was confirmed between the two variables for each treatment (Appendix 7.1). When each single treatment was repeated in different animals, any difference in the rate of penicillin decay in CF was minimal (NS). These nonsignificant differences between repeat experiments (involving all treatments) are illustrated by the low F values (i.e. $F_{18}^3 = 1.79$; Appendix 7.2). The minimal differences observed

in experiments between animals, enabled the construction of a common regression line representing all replicates for each chosen treatment and these were subsequently used to compare the effects of different treatments.

The peak concentration of penicillin observed in CF after topical instillation of the aqueous solution of sodium benzyl penicillin illustrated a greater variation between repeat experiments (i.e. 5-63 iu/ml: Fig 7.1) than that of the ointment form (i.e. 5-12 iu/ml: Figs 7.2, 7.3 & 7.4).

Topical application of sodium benzyl penicillin in an aqueous vehicle (Treatment 9) produced the shortest duration of penicillin concentration above the minimum therapeutic concentration (12.6 ± 1.5 hours: Table 7.1). The average peak concentration of 10 iu/ml was observed immediately after the treatment.

Ointment preparations of all three penicillin salts significantly prolonged ($P < 0.01$) the duration of therapeutic concentration in CF. The durations observed for aqueous soluble sodium benzyl penicillin and the less aqueous-soluble procaine penicillin were similar, when each was applied in the ointment base. The mean durations for sodium penicillin and procaine penicillin were 39.8 ± 2 hour and 37.0 ± 4 hour (Table 7.1) and the approximate peak concentration 7 iu/ml (Fig. 7.2) and 13 iu/ml (Fig. 7.3) respectively.

Benethamine penicillin in the same ointment base showed a significantly extended ($P < 0.01$) duration, the mean of which for four animals was 56 ± 4.5 hours (Table 7.1). The estimated peak concentration of 14 iu/ml was observed immediately after administration.

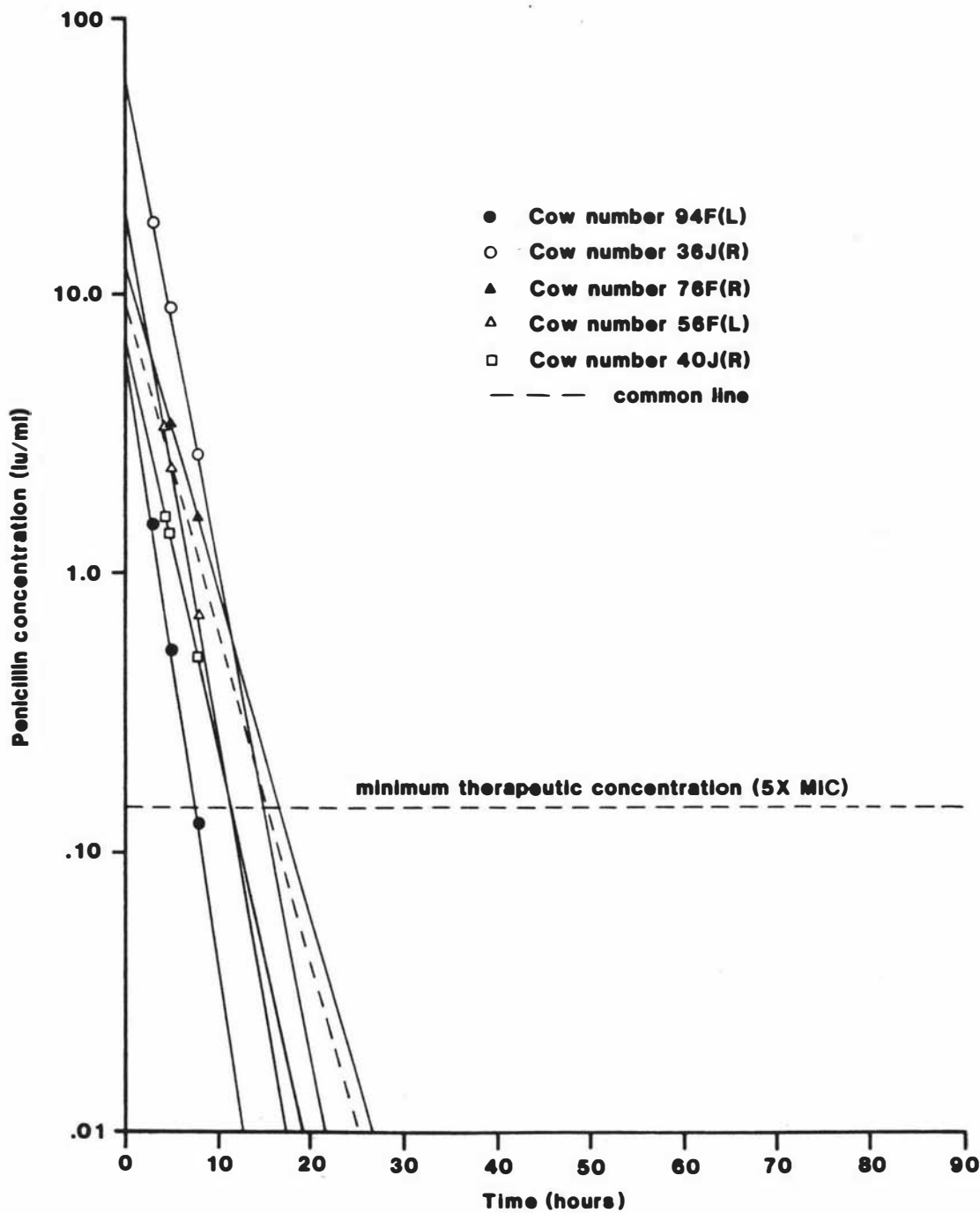


Fig. 7.1 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after topical application of sodium benzyl penicillin in distilled water (5,000 iu in 0.1 ml).

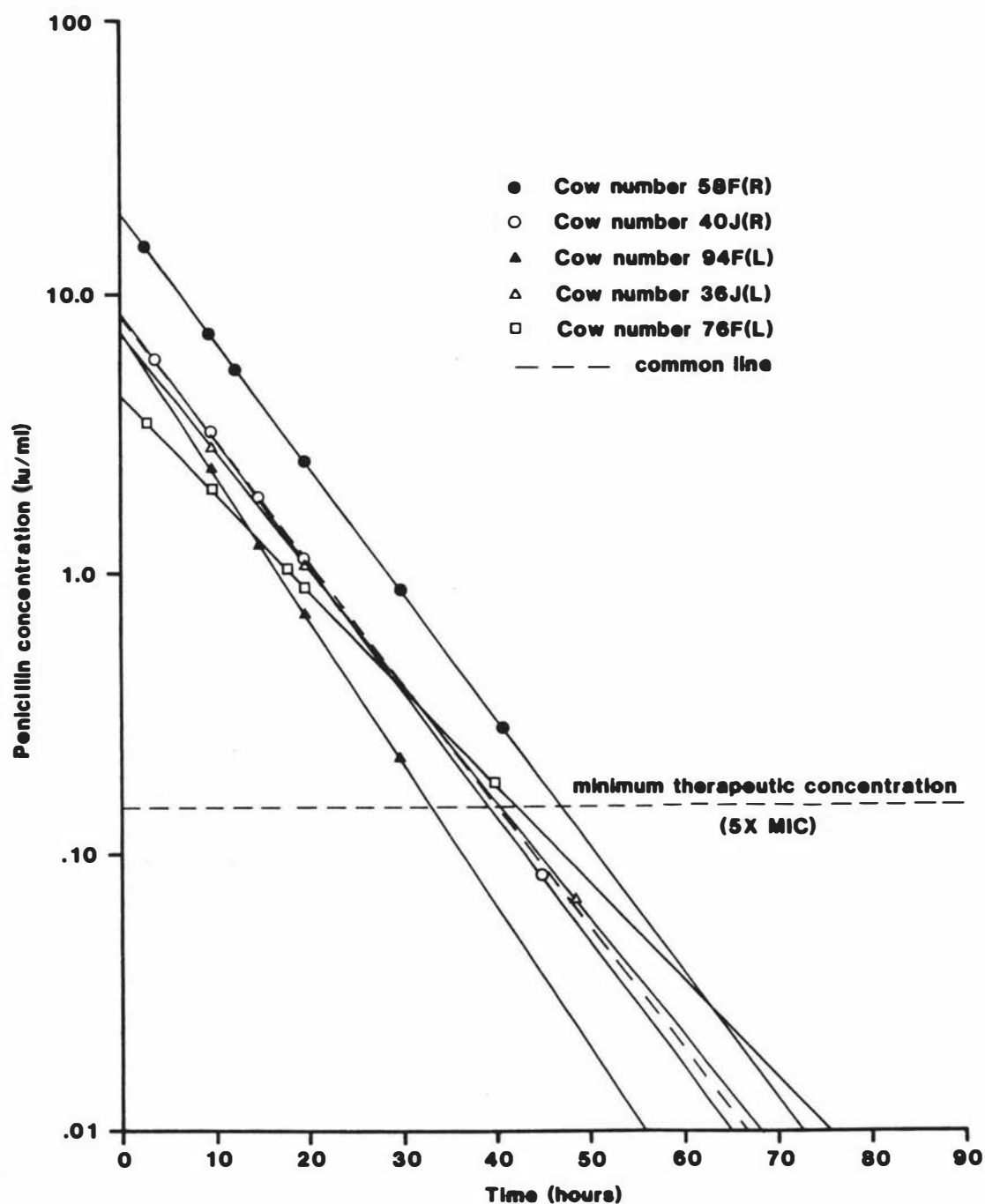


Fig. 7.2 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after topical application of sodium benzyl penicillin in the ointment base (5,000 iu in 0.3 gram).

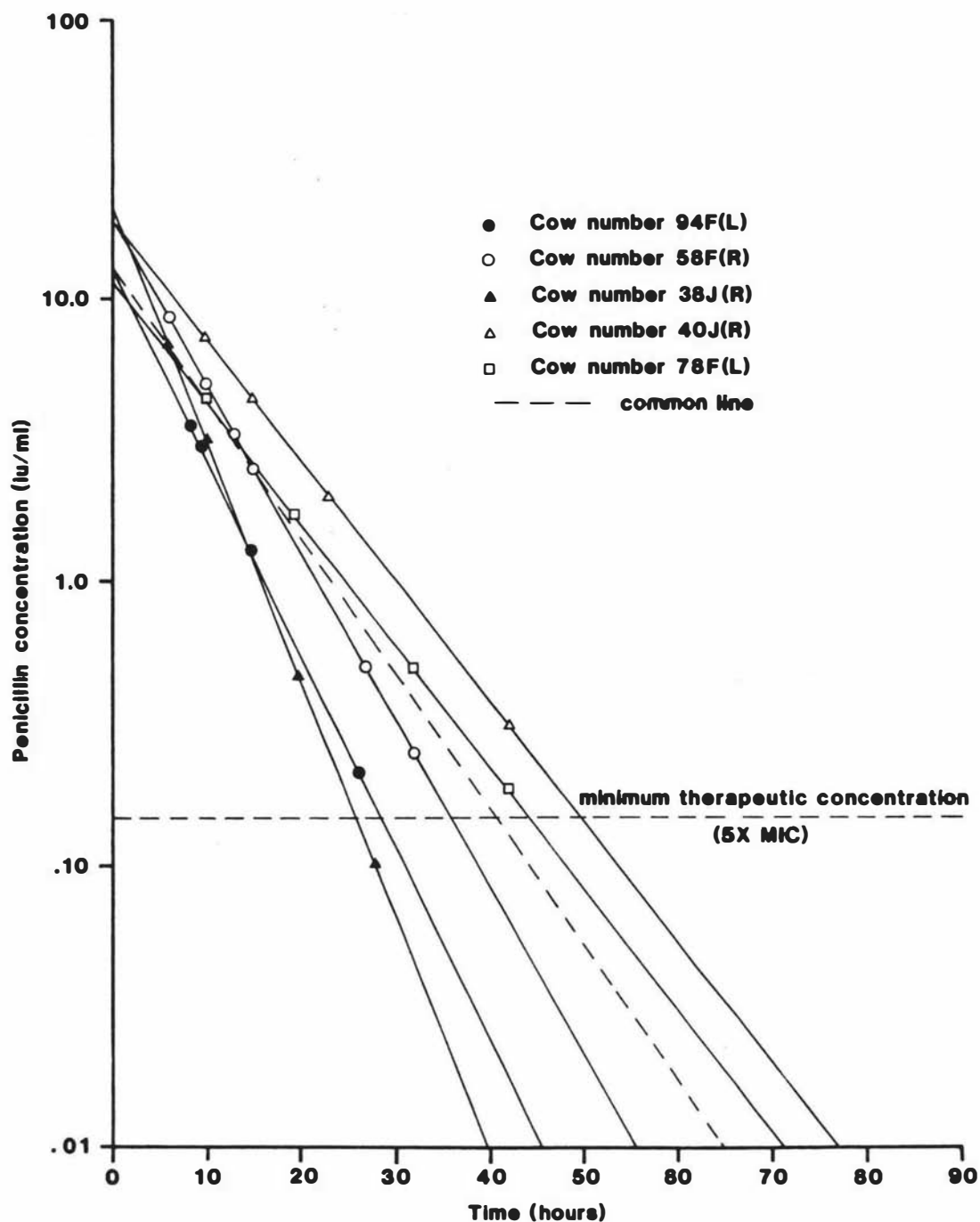


Fig. 7.3 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after topical application of procaine penicillin in the ointment base (5,000 iu in 0.3 gram).

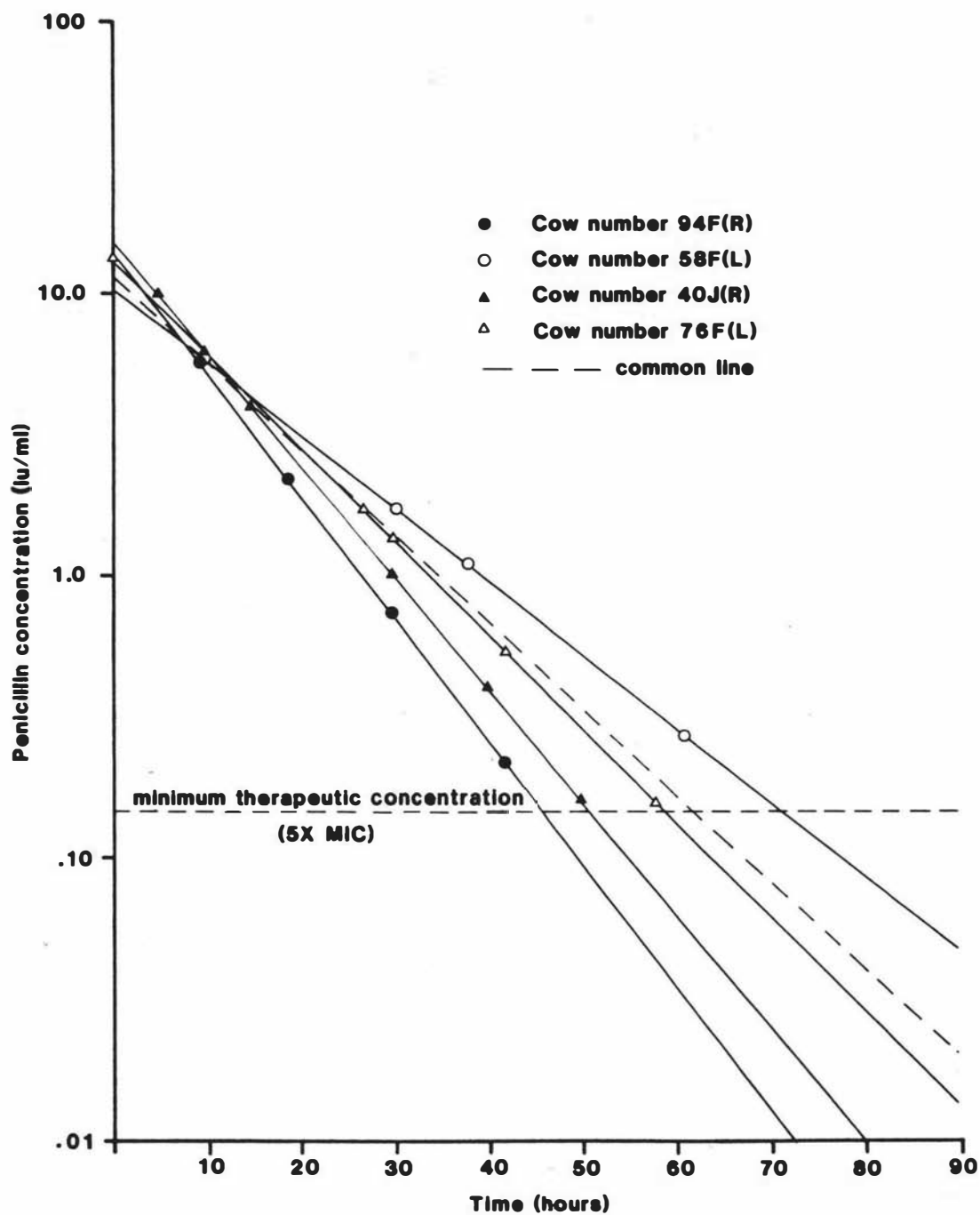


Fig. 7.4 A semilogarithmic plot of individual eye regression lines for penicillin in conjunctival sac fluid against time, after topical application of benethamine penicillin in the ointment base (5,000 iu in 0.3 gram).

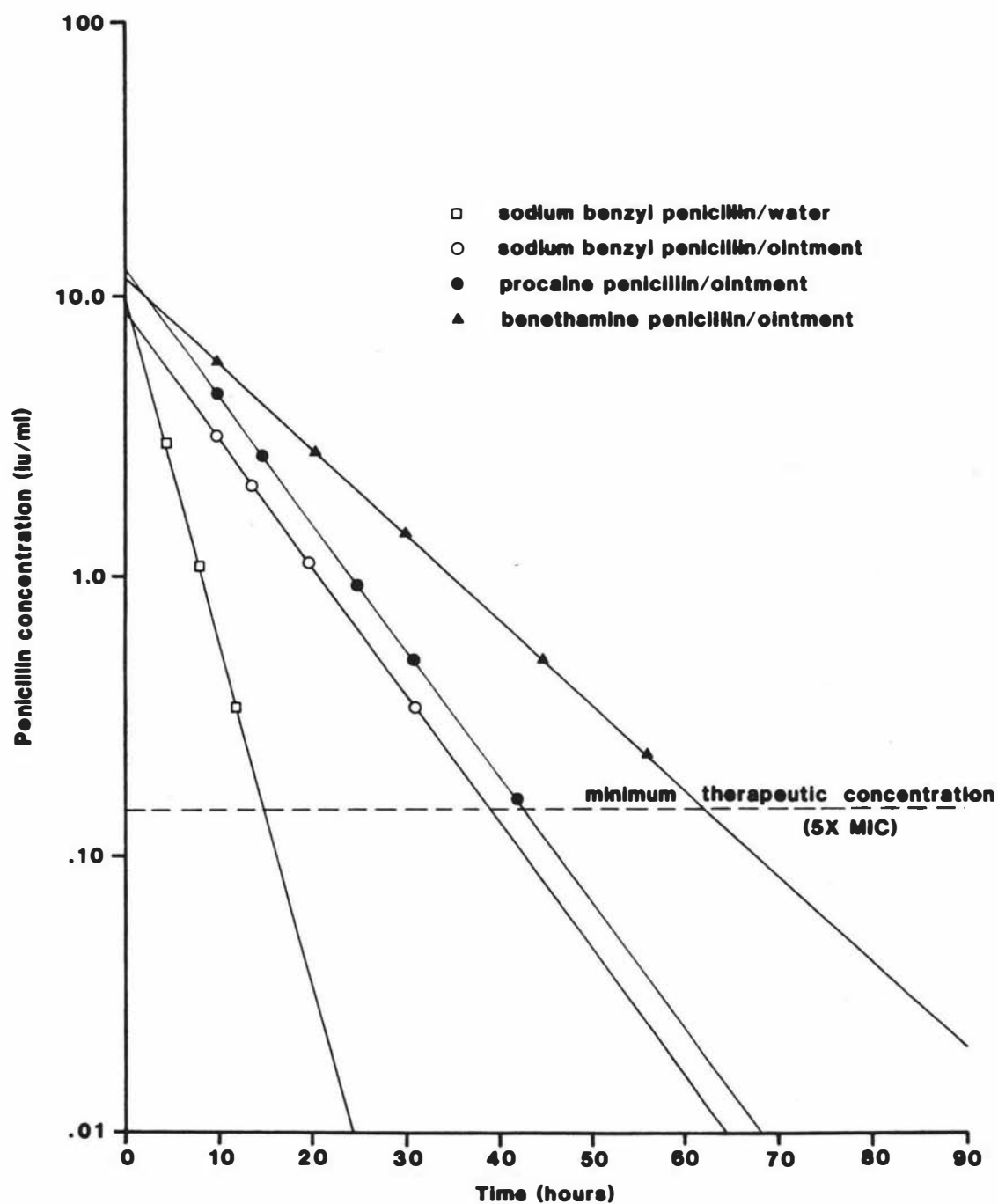


Fig. 7.5 A semilogarithmic plot of common regression lines representing the penicillin concentration in conjunctival sac fluid after topical instillation of different salts of penicillin.

Table 7.1 Comparison of different topical treatments of penicillin using the common regression line of each treatment

TREATMENT number / product / vehicle		Regression coefficient of the common line ($\pm$ 95% confidence limits)	Significance of difference		Duration of therapeutic concentration: $\geq 5 \times$ MIC hours ($\pm$ SEM)
9	sodium benzyl penicillin /distilled water	- .1279 ($\pm$.0248)	**	a	12.6 $\pm$ 1.5205
10	sodium benzyl penicillin /ointment base	- .0440 ($\pm$.008)	NS	b	38.8 $\pm$ 2.1019
11	procaine penicillin /ointment base	- .0492 ($\pm$.0106)	NS	b	37.0 $\pm$ 3.9802
12	benethamine penicillin /ointment base	- .0315 ($\pm$.005)	**	c	56.0 $\pm$ 4.5000

DISCUSSION

The results of this study clearly showed that peak concentrations and duration of therapeutic level were strongly influenced both by the base of the formulation and by the relative water solubility of the penicillin salt used.

The substantial differences in peak concentration between individual cattle eyes observed after topical instillation of sodium benzyl penicillin in aqueous solution, could be attributed to the immediate availability of the drug, in contrast to a slower release from the ointment base. Except for the above-mentioned variability, a minimal difference in peak concentration and duration was observed between repeat experiments using the same treatment on different animals and this enabled the construction of a common regression line for each treatment. Subsequently, comparisons between treatments could be made using such common lines.

The results of all topical instillations, generally illustrated an exponential decay of penicillin in CF (Fig. 7.5). The peak concentrations observed immediately after topical administrations were mostly in the same range; (approximately 10 iu/ml: Fig. 7.5) and this similarity could be attributed to the effect of equivalent dosages that were instilled at the common site. On the other hand the rate of penicillin concentration decline in CF (described by the regression coefficient) and as a result, the duration of a

therapeutic concentration, differed substantially between different penicillin products.

Sodium benzyl penicillin in aqueous solution provided the base line against which all other treatments were compared.

Although the topical application of sodium benzyl penicillin in the aqueous phase produced the shortest duration (12.6 ± 1.5 hours) of all four treatments carried out in this series, even that is considered longer than might have been expected from general information on therapy of eyes. For example, the literature dealing with treatment in human eye diseases and also that concerning treatment of IBK (Faull & Hawksley, 1954) advocates frequent instillations of penicillin, in some cases as often as every 30 minutes (Sorsby, 1960), in order to maintain the desired therapeutic concentration. According to the results of the present study such frequent instillation would be unnecessary. Perhaps some physiological peculiarity of cattle accounts for this longer retention time even though normal tear flow rates for humans; 0.5 - 2.2 $\mu\text{l}/\text{minute}$ (Mishima *et al*, 1966) are substantially slower than for cattle; 9 - 31 $\mu\text{l}/\text{minute}$ (Hoffmann & Spradbrow, 1978).

Assuming that 5,000 iu of penicillin are distributed immediately throughout 100 μl in the bovine CF and that the flow rate is 10 $\mu\text{l}/\text{minute}$ (Hoffmann & Spradbrow, 1978) the concentration of penicillin in CF should have reached the minimum therapeutic concentration in 100 minutes. As it was, in the cattle experiments, the aqueous solution of penicillin persisted for more than 12 hours; therefore any assumption of a simple physical dilution effect

must be incorrect. The volume and dose of penicillin instilled would have been capable of saturating the thin layer of fluid covering the entire conjunctiva and cornea (Adler *et al*, 1971) and therefore other factors concerned with relative solubility or specific binding properties of the penicillin molecules must account for the extended period of therapeutic duration.

For each treatment investigated, the results confirmed that products formulated with the ointment base produced a relatively extended duration of therapeutic concentration: a finding in keeping with those of Linn & Jones (1968): humans, Havener (1974): humans, Hardberger *et al* (1975): rabbits, and Wilson *et al* (1983): rabbits. They reported a direct relationship between the duration of drug concentration in fluids on the surface of the eye, and the viscosity of the vehicle in which the drug was formulated. In addition, the slow liberation of penicillin from an ointment base into the aqueous phase of CF could also account for part of the observed persistence.

The duration of therapeutic concentration produced by the relatively insoluble procaine penicillin (British Pharmacopoeia-Veterinary, 1977) in the ointment base, was similar to that of the far more water-soluble sodium benzyl penicillin when that was incorporated into an ointment base (Fig. 7.5). This similarity suggests that a slowed dissolution of the formulation is more of a determining factor in sustaining a concentration in CF than the oil/water partition coefficient of either penicillin salt.

Benethamine penicillin is chemically defined as N-benzyl- β -phenylethylamine of benzyl penicillin (Nelson *et al*, 1954; Basil *et al*, 1955). The present study illustrated that benethamine penicillin in the ointment form further extended the duration of the therapeutic concentration and that the prolonged effect could be attributed to its relative aqueous insolubility (British Pharmacopoeia-Veterinary, 1977). Such a finding is supported by the recent work of Buswell *et al* (1982) who obtained an extended duration of effect (namely 56 hours) when using benzathine cloxacillin in an oily base.

In general, the extended duration of therapeutic concentrations observed for all topical treatments in this series, could be due partly to the high dose of penicillin which would have saturated the fluid layers covering the mucosae. When such fluid is entrapped in deep fornices, penicillin in solution may take some time to reach equilibrium with the same material in the precorneal tear film so that there may be substantial delays before significant amounts disappear into the lachrymal duct. In addition, the lipophilic nature of the tear film is likely to retain liquid paraffin on its surface (Ehlers, 1965b) and this effect could have prolonged the presence in the CF of penicillin, had it been applied in an ointment base. The lipophilic nature of the corneal epithelium itself (Lee *et al*, 1983) has been shown to retain drugs which are in a lipid (ointment) vehicle. Thereafter a slow release of the active ingredient into the tear film and eventually CF, could be expected (Nagataki & Mishima, 1980; Wyman, 1975).

A similar extended duration associated with the use of ointment in other species was reported by Massey *et al* (1976) and Nagataki & Mishima (1980). Finally, the fine structure of the lining mucosae, such as crypts, infoldings (Carroll & Kuwabara, 1968), microvilli (Pedler, 1962; Pfister & Burstein, 1976) and goblet cells (Wanko *et al*, 1964) may be considered as contributory factors in retarding the movement of any molecules not in solution and therefore they too would contribute in delaying removal of the drug from the site.

The various topical treatments used appeared to be quite safe. Neither the bland ointment alone nor an insoluble salt of penicillin in an ointment base caused any visible clinical changes. Excessive lachrymation observed momentarily just after topical administration of medication could be due to the reflex nerve stimulation as suggested by Mishima *et al* (1966). Temporary blurring of vision associated with the use of eye ointments is considered undesirable in human therapeutics (Adler *et al*, 1971; Evans, 1971). However, in the treatment of IBK, such a film over the cornea could be advantageous if it proved to be a barrier to ultra violet rays striking the exposed surface of the bovine eye. However, where open corneal wounds are involved (Wyman, 1975), ointments are contraindicated because they may predispose to severe inflammatory reactions. Some stages of IBK would be associated with severe corneal changes of this type and accordingly ointments ought not to be used under such circumstances.

With the exception of such extreme cases, the topical instillation of chemotherapeutic substances, particularly as ointments, is considered a satisfactory method of IBK control (Faull & Hawksley, 1954; Gallagher, 1954; Jackson, 1954; Cooper, 1960; Ellis & Barnes, 1961; Pugh, 1978; Buswell *et al*, 1982; Nyack & Padmore, 1982) and the observations on drug distribution made in the present study tend to support this contention. For a number of antibacterial agents including penicillin, far more data on the elimination of *M. bovis* infections following an optimum treatment schedule, are needed. Such information would only be obtainable from a number of well designed clinical trials.

CHAPTER 8

GENERAL DISCUSSION

Moraxella bovis, the causative organism of infectious bovine keratoconjunctivitis (IBK) is sensitive to many different antimicrobial products (Pugh & McDonald, 1977; Webber et al, 1982). However, the distribution of these chemotherapeutic substances, particularly in terms of the magnitude and the duration of drug concentration in the conjunctival sac is largely unknown. It was for this reason and because of the important nature of the disease and the already extensive use of antibiotics in the treatment, that the work reported in this thesis was undertaken. Further, the dearth of knowledge concerning the absorption and duration of any of these chemotherapeutic agents necessitated the use of a relatively familiar and well tried antibiotic. Accordingly penicillin was chosen although it is not necessarily the antibiotic of choice in the treatment of either *M. bovis* infections or indeed any bacterial condition of the eye. However, it is possible to obtain a range of penicillin products all containing the same active principle and differing only in either the salt, or components of the formulation. Thus, individual experiments of this project were concerned with determining the penicillin concentration in CF after various penicillin products had been administered by different routes. It was decided to investigate the use of a single dose treatment rather than repeated ones, because the aim of this study was to obtain information which would be useful in deriving

a practical treatment schedule for IBK, in the field.

As the project developed, it became clear that the method used for obtaining CF samples was of prime importance. The collection procedure had to be nonirritating to the eye. In addition, the animal itself had to be relatively undisturbed in order that local and systemic nerves were not stimulated to produce lachrymal fluid. It is known that lachrymal fluid which has been secreted by distressed animals (Best & Taylor, 1973) contains endogenous antibacterial substances (Morrison & Allen, 1966) which might invalidate the results of penicillin assays using a biological system. Animals had to be trained to the collecting procedure as any form of chemical restraint may have invalidated for one reason or another results of the bioassay. Under such circumstances and using blunted capillary tubes, it proved possible to collect uncontaminated samples with relative ease, and this satisfied the requirements of the assay system.

The selection of a bioassay technique for penicillin estimations was based on the widespread use of such systems in the determination of antibiotics in a variety of biological fluids (Simon & Yin, 1970; Kavanagh, 1972). Because of the small volume of the test sample available, an agar-well diffusion assay technique was chosen and certain modifications had to be made (Chapter 2; Section 2.1) in order to obtain sufficient sensitivity in the specified range of activity. The large assay plate in which *B. subtilis* was incorporated into the agar as the indicator

organism, provided a high degree of accuracy and reproducibility throughout.

While a bioassay system can be used to detect penicillin at very low concentrations, in this study attention was restricted to a range of levels at which it could be predicted with confidence that a bactericidal effect on *M. bovis* would be evident. Accordingly, a 'minimum therapeutic concentration' in CF was adopted. This figure was based on the MIC for penicillin against local strains of *M. bovis* and had superimposed on that a liberal safety factor to make allowance for local conditions in the conjunctival sac. From such derived minimum therapeutic concentrations for various treatment forms, it was possible to predict for each product, the period of time during which the therapeutic concentration of penicillin in CF would be exceeded (Fig. 4.1; page 66).

The systemic route of drug administration is not uncommonly used in treatment of eye diseases (Havener, 1974) and therefore intramuscular or subcutaneous injections were included amongst the other more practical means of administration investigated in this study. The aim of these preliminary series of experiments was to obtain basic information on penicillin disposition in CF; in terms of the speed of distribution, magnitude of concentration and duration of levels after different treatments had been given. In order to illustrate disposition throughout the body, data dealing with penicillin profiles in serum were processed according to an appropriate pharmacokinetic model, but penicillin concentrations

in CF were always evaluated using regression analysis. The results indicated that systemic injections of either sodium benzyl penicillin or procaine penicillin are unlikely to be successful in the treatment of IBK. It was observed however, that penethamate hydriodide could be of some value in situations where systemic routes have to be relied upon. Although potentially effective, the high cost of penethamate hydriodide would probably prohibit its general use under extensive farming conditions.

Even though both eyes of an animal may be treated by a single drug administration, systemic routes cannot be considered the best approach to therapy as the site of administration is too far removed from the focus of infection. The distance involved, the dilution effect and the resistance imposed by biological membranes tend to be limiting factors in distribution and consequently, to obtaining an adequate concentration of drug at the site of infection. In contrast, any form of local administration, is likely to be more effective because it is now known that it produces the desired therapeutic concentration in superficial tissues of the eye (Chapters 6 & 7).

Procaine penicillin given by subconjunctival injection is commonly employed in the treatment of IBK in New Zealand. The results of studies reported in this thesis tend to support the field practice because adequate drug concentrations were maintained in CF for over 48 hours. By administering procaine penicillin through the skin of the eye lid and leaving the conjunctiva intact, it was

possible to extend that duration of effect. However in using the skin route, the animal's resistance to handling and general apprehension were increased as the needle had to be directed through a greater number of tissues including the tarsal plate of the eye lid.

The topical instillation of penicillin or its products into the human eye is not generally recommended (Ellis, 1977) because of the risk of hypersensitivity reactions. However, the route is considered safe in veterinary therapeutics (Brightman, 1980), and topical administration of penicillin as well as other drugs has been widely used in the treatment of IBK (Faull & Hawksley, 1954). In view of the widely held belief that subconjunctival injections produce extended periods of therapeutic effect, it was surprising to find that the topical use of either sodium or procaine penicillin in the ointment form gave periods of effect that were not significantly shorter.

Benethamine penicillin in the ointment form further extended the duration of a therapeutic concentration. In this respect, the persistence of penicillin activity was similar to durations reported by Buswell *et al* (1982) who had compared the topical instillation of benzathine cloxacillin in an oil formulation with subconjunctivally injected amoxycillin. These workers confirmed the effectiveness of topical instillation in the treatment of IBK, by a field trial in which they observed a rapid clearance of clinical signs and conjunctival infection.

Although clinical trials were not carried out in the current study, there is every indication that instillation of penicillin would be a feasible curative measure for IBK. The topical treatment could easily be carried out by farmers and repeated applications if required should not cause any reaction. Even in the event of serious outbreaks, treatment begun by veterinarians using subconjunctival injections could be continued by farmers using topical ointments. Nevertheless, as a prerequisite to any authoritative statement on a treatment strategy for IBK, the effectiveness of the currently studied treatments needs to be confirmed by controlled clinical trials.

Any investigations made on infected eyes are unlikely to yield results comparable to the current series of experiments using normal animals. In the first place different stages of IBK in different eyes would produce variable degrees of increased lachrymation and secondly, endogenous antibacterial substances and inflammatory cells might well invalidate the assay system presently used. However, in preliminary experiments involving four animals which were infected with IBK, the subconjunctival injection of 300,000 iu of procaine penicillin in 1 ml produced a rate of decline in CF that was not significantly different from that of clinically normal animals (Chapter 6: Treatment 8).

No attempt was made in the current study to include any other products that have been tried in the treatment of IBK. Penicillin is certainly cheap, safe and apparently effective under New Zealand

conditions, but that situation may not prevail. In countries in which IBK has been recognized for many years, there have been reports of developing resistance against penicillin by some strains of *M. bovis* (Pugh & McDonald, 1977; Webber *et al* , 1982). This state of affairs could possibly be attributable to the long-term exposure of *M. bovis* to penicillin. To avoid similar developments in New Zealand, the sensitivity of a number of *M. bovis* isolates should be investigated on a regular basis. There is an obvious need too for a review of the non-antibiotic antibacterial products that might prove useful in the treatment of this disease. Work needs to be undertaken with such products to ensure that adequate drug levels are sustained in CF, and that *M. bovis* infection will be eradicated by the concentration achieved. Simplified methods of administration must continue to be sought too, because at the present time, it is this aspect of treatment which presents the greatest single limitation to the control of IBK amongst groups of grazing cattle.

Infectious bovine keratoconjunctivitis is considered to be a disease of recent origin in New Zealand (Harris *et al*, 1980). The present work has shown the sensitivity of some local isolates of *M. bovis* towards penicillin and while this antibiotic continues to be used for the treatment of IBK, every effort should be made to see that the schedule of medication produces the optimum antibacterial effect. As a result of pharmacokinetic data obtained during the present study, it is now possible to devise treatment schedules for each of the penicillin products tested.

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APPENDIX 2.1

Assay Medium No. 5

Peptone	6 gram
Yeast extract	3 gram
Beef extract	1.5 gram
Agar	15.0 gram
Distilled water to make	1000 ml
pH 7.8-8.00 after sterilization	
(Grove & Randall, 1955; Arret <i>et al</i> , 1971).	

APPENDIX 2.2

ESTIMATION OF PENICILLIN CONCENTRATION IN THE SAMPLE OF
CONJUNCTIVAL SAC FLUID BY REFERENCE TO A STANDARD CURVE.

An example of a calculation to find the line of best fit using
least square analysis (Snedecor & Cochran, 1972);

Diameter of the inhibitory zone (mm)	Penicillin concentration (iu/ml)	XY	X ²
22.735	5.00	15.8918	516.88
17.306	1.00	0	299.49
8.410	.10	-8.4100	70.73
7.732	.083	-8.3575	59.784

X = zone of inhibition (mm)

Y = log₁₀ penicillin concentration (iu/ml)

The regression coefficient (b) was calculated using the formula,

$$b = \frac{\sum XY - n\overline{XY}}{\sum X^2 - n\overline{X^2}}$$

Having calculated the regression coefficient, the standard curve of
penicillin was constructed which represented the line of best fit.

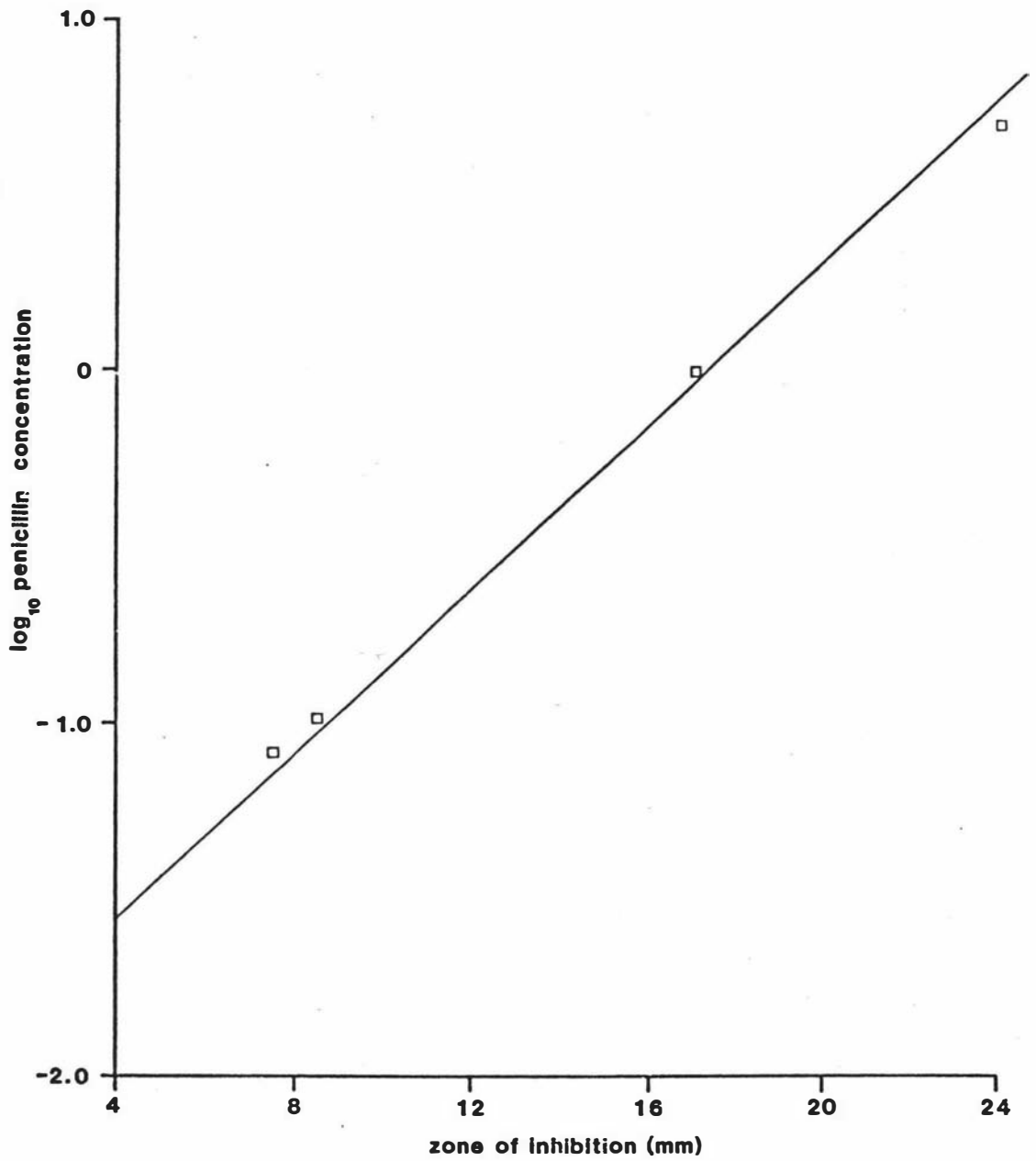
For selected X values, corresponding $\hat{Y}$ values can be calculated
according to the formula,

$$\hat{Y} - \bar{Y} = b (X - \bar{X}) \text{ (Snedecor \& Cochran, 1972)}$$

This example is expressed graphically in Appendix 2.3.

Using the constructed standard curve, penicillin concentration
($\hat{Y}$) in CF samples was estimated.

APPENDIX 2.3



A STANDARD CURVE USED TO PREDICT THE $\log_{10}$ PENICILLIN CONCENTRATION FOR AN OBSERVED ZONE OF INHIBITION

APPENDIX 2.4

Substitute for conjunctival sac fluid

Sodium chloride	0.8541 gram
Potassium chloride	0.0313 gram
Methyl cellulose	0.0010 gram
Blood cells	0.10 ml

dissolved in distilled water to make 100 ml

pH was adjusted 7.0 - 7.3

(Thaysen & Thorn, 1954; Pedersen, 1973).

APPENDIX 2.5

(A) Regression analysis of two series of penicillin dilutions

Penicillin dilution series were prepared in either distilled water or CF substitute. An example of two series of results obtained on a single assay plate is given below.

Results of the bioassay when penicillin is dissolved in distilled water:

Penicillin concentration (iu/ml)	Zone of inhibition (mm)				
	Y	X			
10.0	1.0	25.20	24.80	24.55	24.40
5.0	.6989	23.00	22.90	21.80	22.00
1.0	0	18.15	16.90	17.70	17.50
.10	-1.0	9.10	9.30	9.10	9.20
.083	-1.0809	8.50	8.20	8.00	7.30

Results of the bioassay when penicillin is dissolved in the conjunctival sac fluid substitute:

Penicillin concentration (IU/ml)	Zone of inhibition (mm)				
	Y	X			
10.0	1	24.25	23.90	24.45	24.00
5.0	.6989	21.46	22.75	22.15	21.60
1.0	0	17.50	17.50	17.50	17.20
.10	-1.0	-	9.0	9.15	9.10
.083	-1.0809	8.0	7.9	8.05	7.60

The regression lines for each series, representing the relationship

between penicillin concentration and zone of inhibition were calculated using the following formulae (Snedecor & Cochran, 1972).

$$\Sigma x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{n}$$

$$\Sigma y^2 = \Sigma Y^2 - \frac{(\Sigma Y)^2}{n}$$

$$\Sigma xy = \Sigma XY - \frac{\Sigma X \cdot \Sigma Y}{n}$$

where X = zone of inhibition (mm)

Y = log₁₀ penicillin concentration (iu/ml)

(B) Analysis of variance of each regression line

The following table is an example of calculations used in testing the significance of the regression equation and its linearity.

Source of variance	df	SS	MS	F	Significance
Original	19	922.807			
due to regression (slope)	1	562.5745	562.574 (B ²)		
deviation from regression (scatter about line)	18	5.4362	.3020 (S ²)	1862	**

df = degrees of freedom

SS = sum of square deviation from regression

MS = mean square deviation from regression

S² = error variance about the line

The ratio B²/S² (F value) tests the null hypothesis;
(i.e. dependence of inhibitory zones upon penicillin concentration.
Thus β = 0). The F value rejects the null hypothesis (P < .01)
and confirms the dependence of Y upon X.

(C) Comparison of two regression lines

The following is an example of values that were used to determine the significance of difference between dose-response curves derived from two penicillin dilution series made up in different solvents.

Source of variance	df	Σx^2	Σxy	Σy^2	b	deviation from regression		
						df	SS	MS
penicillin in distilled water	19	14.5111	115.3779	922.807	7.95	18	5.4362	.3020
penicillin in CF substitute	18	13.6126	105.3788	820.612	7.74	17	4.8464	.2551
for individual regression lines						35	10.2826	.2938 ^B
for the common regression line	37	28.1237	220.7567	1743.419	7.85	36	10.5915	.2942
difference between slopes						1	.3089	.3089 ^A

$$F_{35}^1 = A/B = 1.0514 \text{ (NS)}$$

b = regression coefficient ($\frac{\Sigma xy}{\Sigma x^2}$)

df = degrees of freedom

SS = sums of squares

MS = mean squares

The hypothesis that there is no difference between the slopes for the two penicillin dilution series was upheld.

APPENDIX 2.6

EXAMPLES OF STANDARD ERRORS OF SOME PREDICTED PENICILLIN CONCENTRATIONS
OBTAINED USING THE BIOASSAY TECHNIQUE

$X - \bar{X}$	$\hat{S}_\mu$	$X - \bar{X}$	$\hat{S}_\mu$	$X - \bar{X}$	$\hat{S}_\mu$
<u>Treatment 10.1</u>		<u>Treatment 11.1</u>		<u>Treatment 12.3</u>	
.4235	.1515	.4442	.1644	.3938	.1323
.3582	.1327	.3510	.1361	.3479	.1200
.2393	.1008	.3474	.1350	.3210	.1129
.2113	.0941	.2458	.1063	.2379	.0921
.0843	.0701	.1941	.0932	-.1476	.0728
.0033	.0011	.1293	.0793	.1807	.0793
.0218	.0650	.1061	.0752	.1885	.0810
.0111	.0647	.0157	.0663	-.1526	.0737
				-.1018	.0652
				.0094	.0575
				-0.0770	.0620
				-.0647	.0607
				.0501	.0594
<u>Treatment 10.2</u>		<u>Treatment 11.2</u>			
.4474	.1627	.4404	.1628		
.3227	.1277	.3305	.1297		
.2141	.1007	.2432	.1055		
-.0063	-.0728	-.0538	.0685		
		.0789	.0713		
		<u>Treatment 12.2</u>		<u>Treatment 12.1</u>	
		.4296	.1503	.4150	.1549
		.3541	.1284	.3741	.1424
		.2932	.1114	.3132	.1243
		.0638	.0628	.1198	.0759
				.0712	.0685
				.0107	.0642
				-.0372	.0653
				-.0992	.0724

$X - \bar{X}$ = deviation from mean (x)
 $\hat{S}_\mu$ = sample standard deviation of $\hat{Y}$ as an estimate
(standard error of predicted penicillin concentration)

APPENDIX 4.1

Degree of correlation between penicillin dilution factor and the diameter of the inhibitory zone; when dilutions were prepared either in distilled water or Heart Infusion Broth.

Source of difference plate identity/diluent		Coefficient of determination (r^2)	F	df	Significance
21/3	dw	.9952	50	1:14	**
24/3	dw	.9943	90	1:13	**
27/3	dw	.9848	48	1:10	**
21/3	HIB	.9899	267	1:26	**
24/3	HIB	.9947	320	1:26	**
27/3	HIB	.9950	159	1:18	**
27/3	HIB	.9951	234	1:22	**

dw : distilled water

HIB : Heart Infusion Broth

df : degrees of freedom

APPENDIX 4.2

Confirmation of penicillin stability after overnight incubation
in Heart Infusion Broth

Source of difference plate identity/diluent		Regression coefficient ($\pm$ se)	F	df	Significance
21/3	dw	.1224 ($\pm$.0329)	1.0294	1:40	NS
21/3	HIB(fresh)	.1285 ($\pm$.0016)			
24/3	dw	.1141 ($\pm$.0242)	0.2319	1:39	NS
24/3	HIB(after incubation)	.1163 ($\pm$.0129)			
27/3	dw	.1269 ($\pm$.0374)	0.4844	2:50	NS
27/3	HIB(after incubation)	.1167 ($\pm$.0185)			
27/3	HIB(fresh)	.1307 ($\pm$.0170)			
27/3	dw(fresh)	.1253 ($\pm$.0251)	0.5318	1:40	NS
	dw(after incubation)	.1183 ($\pm$.0143)			

dw : distilled water

HIB : Heart Infusion Broth

se : standard error

df : degrees of freedom

APPENDIX 5.1

COMPARTMENT MODELS USED TO CALCULATE DISPOSITION KINETICS
OF PENICILLIN IN BOVINE SERUM.

Treatment 1: Sodium benzyl penicillin (20,000 iu/kg) I.V.

The two compartment open model was used to evaluate intravenous administration of sodium benzyl penicillin, using the method described by Baggot (1977). The drug concentration (iu/ml) in serum is expressed as a function of time according to the equation

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where B = intercept of back extrapolated slope of elimination phase
 A = intercept of back extrapolated slope of distribution phase
 β = slope of elimination phase
 α = slope of distribution phase
 e = base of natural logarithm (ln)

Iterative linear regression lines were then calculated starting from the terminal phase of elimination, using the equation

$$Y = a + bX$$

where Y = natural logarithm of penicillin concentration in serum (iu/ml)
 and X = time (hours)

The line of best fit for α and β phases was selected based on the highest F value, using the equation

$$F = \frac{(r-2)r^2}{(1-r^2)} \quad (\text{MacDiarmid, pers. comm.})$$

where r = coefficient of correlation.

The residual data points representing the distribution phase, were obtained by the feathering technique (Baggot, 1977). Thereafter the

following disposition kinetics were calculated;

a) the distribution rate constant (from peripheral to central compartment),

$$k_{21} = \frac{A\beta + B\alpha}{A+B} \quad (\text{Baggot, 1977})$$

b) The elimination rate constant,

$$k_{el} = \frac{\alpha\beta}{k_{21}} \quad (\text{Baggot, 1977})$$

c) The distribution rate constant (from central to peripheral compartment),

$$k_{12} = \alpha + \beta - k_{21} - k_{el} \quad (\text{Baggot, 1977})$$

d) The half-life of a drug, which is the time taken for the serum concentration of a drug to decline by 50% during the elimination phase

$$t_{1/2} = \frac{\ln 2}{k_{el}} \quad (\text{Baggot, 1977})$$

e) The apparent volume of distribution, which is hypothetical value defined as the volume of body water required to contain the amount of drug in the body if it were uniformly present in the same concentration in which it is in the blood (Gibaldi et al, 1969; Notari, 1973; Baggot, 1977; Curry, 1977) at any time after distribution equilibrium has been attained.

$$Vd = \frac{\text{dose (I.V.) } k_{el}}{\beta \cdot C_p^0}$$

$$C_p^0 = A + B$$

Treatment 2: Procaine penicillin (20,000 iu/kg) I.M.

A two compartment open model was used to evaluate the use of procaine penicillin administered by the intramuscular route. The absorption and elimination phases were calculated according to the method used previously.

The absorption rate constant,

k_{ab} = slope of the regression line which represents
the absorption phase (Ritschel, 1976).

Treatments 3 & 4: Penethamate hydriodide (20,000 iu/kg)

The single compartment open model following extravascular administration (Ritschel, 1976) was used to describe the disposition kinetics of penethamate hydriodide injected by the intramuscular or subcutaneous route.

The drug concentration in serum as a function of time was expressed as,

$$C_p = Be^{-\beta t} \quad (\text{Baggot, 1977})$$

$$C_p^0 = B \quad (\text{Ritschel, 1976})$$

As penethamate hydriodide is a highly lipid soluble ester of penicillin, only two phases namely, absorption and elimination phases can be defined. The method involved in calculating the elimination phase was the same as that used for Treatment 1. The regression line calculated from residual data points represents the absorption phase, the slope of which represents the absorption rate constant (k_{ab}).

APPENDIX 5.2

THE CORRELATION BETWEEN PENICILLIN CONCENTRATION IN CONJUNCTIVAL
SAC FLUID WITH TIME, AFTER DIFFERENT SYSTEMIC TREATMENTS

Treatment/ animals/eyes	Coefficient of determination	F ratio	df	Significance
<u>sodium benzyl penicillin, 20,000 iu/kg (I.V.)</u>				
38J(R)	.9368	89	1:6	**
38J(L)	.9547	126	1:6	**
78J(R)	.9645	136	1:5	**
78J(L)	.9122	62	1:5	**
40J(R)	.9038	65	1:6	**
40J(L)	.8438	22	1:3	*
70J(R)	.6813	12	1:8	**
70J(L)	.6606	17	1:6	**
<u>procaine penicillin, 20,000 iu/kg(I.M.)</u>				
total dosage at a single site				
70F(R)	.8488	57	1:2	*
70F(L)	.9984	3030	1:3	**
40J(R)	.8488	11	1:3	**
40J(L)	.9983	4000	1:3	**
divided dosage (two sites):				
40J(R)	.8136	10	1:6	*
40J(L)	.7586	29	1:6	**

Appendix 5.2 continued

Treatment/ animals/eyes	Coefficient of determination	F ratio	df	Significance
<u>penethamate hydriodide, 20,000 iu/kg (I.M.)</u>				
40J(R)	.3672	9	1:13	*
40J(L)	.2601	5	1:13	*
70F(R)	.7983	21	1:10	**
70F(L)	.6103	53	1:11	**
78J(R)	.6432	11	1:5	*
78J(L)	.6432	11	1:5	*
79J(R)	.8987	53	1:5	**
79J(L)	.9370	58	1:4	**
38J(R)	.5837	18	1:6	**
38J(L)	.7618	16	1:6	**
<u>penethamate hydriodide, 20,000 iu/kg (S.Ct)</u>				
40J(R)	.7002	14	1:5	*
40J(L)	.8755	19	1:6	**
70J(R)	.6754	10	1:6	*
70J(L)	.7508	45	1:6	**
79J(R)	.6851	16	1:9	**
79J(L)	.8254	25	1:9	**
78J(R)	.6610	21	1:6	**
78J(L)	.7327	15	1:6	**
36J(R)	.5069	11	1:9	**
36J(L)	.8208	35	1:9	**
<u>penethamate hydriodide, 10,000 iu/kg (I.M.)</u>				
40J(R)	.8100	30	1:6	**
40J(L)	.6336	14	1:7	**
79J(R)	.9980	129	1:3	**
79J(L)	.8593	18	1:3	*
78J(R)	.8308	22	1:3	*
78J(L)	.6034	61	1:3	**
36J(R)	.9197	23	1:2	*
36J(L)	.9683	149	1:3	**

APPENDIX 5.3

HOMOGENEITY OF REGRESSION LINES DERIVED FROM DIFFERENT
ANIMALS WHICH RECEIVED THE SAME SYSTEMIC TREATMENT

Treatment/ animal identity	Regression coefficient	95% confidence limits: tSb	Significance of difference
<u>sodium benzyl penicillin, 20,000 iu/kg (I.V.)</u>			
38J(R)	-.3990	± .1174	NS
38J(L)	-.3500	± .0763	NS
78J(R)	-.3460	± .0764	NS
78J(L)	-.3980	± .1296	NS
40J(R)	-.3410	± .1028	NS
40J(L)	-.2390	± .1639	NS
70J(R)	-.2518	± .1404	NS
70J(L)	-.2689	± .0862	NS
			$F_{51}^7 = 0.6132(\text{NS})$
<u>procaine penicillin, 20,000 iu/kg (I.M.)</u>			
total dosage at a single site:			
70F(R)	-.0260	± .0200	NS
70F(L)	-.0310	± .0280	NS
40J(R)	-.0400	± .0149	NS
40J(L)	-.0270	± .0153	NS
			$F_{15}^3 = 0.7836(\text{NS})$
divided dosage at two sites:			
40J(R)	-.0380	±.0940	NS
40J(L)	-.0420	±.0040	NS
			$F_{12}^1 = 1.222(\text{NS})$

Appendix 5.3 continued

Treatment/ animal identity	Regression coefficient	95% confidence limits: tSb	Significance of difference
<u>penethamate hydriodide, 20,000 iu/kg (I.M.)</u>			
40J(R)	-.0215	± .0158	NS
40J(L)	-.0158	± .0158	NS
70F(R)	-.0140	± .0107	NS
70F(L)	-.0340	± .0269	NS
78J(R)	-.0222	± .0078	NS
78J(L)	-.0160	± .0058	NS
79J(R)	-.0398	± .0133	NS
79J(L)	-.0297	± .0149	NS
38J(R)	-.0143	± .0108	NS
38J(L)	-.0181	± .0113	NS
			⁹ F ₈₂ = 1.7206 (NS)
<u>penethamate hydriodide, 20,000 iu/kg (S.Ct)</u>			
40J(R)	-.0271	± .0204	NS
40J(L)	-.0403	± .0141	NS
70J(R)	-.0241	± .0154	NS
70J(L)	-.0320	± .0184	NS
79J(R)	-.0217	± .0111	NS
79J(L)	-.0177	± .0061	NS
78J(R)	-.0144	± .0019	NS
78J(L)	-.0170	± .0113	NS
36J(R)	-.0196	± .0139	NS
36J(L)	-.0106	± .0067	NS
			⁹ F ₈₁ = 1.3165(NS)
<u>penethamate hydriodide, 10,000 iu/kg (I.M.)</u>			
40J(R)	-.0474	± .0210	NS
40J(L)	-.0266	± .0170	NS
79J(R)	-.0685	± .0020	NS
79J(L)	-.0650	± .0426	NS
78J(R)	-.0639	± .0530	NS
78J(L)	-.0637	± .0921	NS
36J(R)	-.0560	± .0500	NS
36J(L)	-.0579	± .0150	NS
			⁷ F ₃₀ = 1.3965(NS)

APPENDIX 6.1

THE CORRELATION BETWEEN PENICILLIN CONCENTRATION IN CONJUNCTIVAL SAC FLUID WITH TIME, AFTER SUBCONJUNCTIVAL INJECTIONS OF PROCAINE PENICILLIN

Treatments/ animals/eyes	Coefficient of correlation	F ratio	df	Significance
<u>6 x 10⁵ iu through skin</u>				
40J(R)	-.8228	20.96	1:9	**
40J(L)	-.9295	63.57	1:9	**
79J(R)	-.9660	181.69	1:12	**
79J(L)	-.7963	22.52	1:12	**
78J(R)	-.8845	32.35	1:8	**
<u>6 x 10⁵ iu through conjunctiva</u>				
40J(R)	-.7863	25.92	1:15	**
36J(L)	-.9595	92.78	1:7	**
79J(L)	-.9774	64.24	1:2	*
78J(R)	-.9941	586.23	1:6	**
79J(L)	-.9768	125.02	1:5	**
<u>3 x 10⁵ iu through conjunctiva</u>				
40J(L)	-.7385	72.38	1:2	*
79J(R)	-.9831	115.46	1:3	**
79J(L)	-.9913	226.95	1:3	**
78J(R)	-.9376	43.64	1:5	**
78J(L)	-.9536	50.14	1:3	**

APPENDIX 6.2

HOMOGENEITY OF REGRESSION LINES DERIVED FROM DIFFERENT EYES WHICH RECEIVED SIMILAR SUBCONJUNCTIVAL INJECTIONS

Source of difference animals/eyes	Regression coefficient	95% confidence limits: tSb	Significance of difference
<u>6 x 10⁵ iu through skin</u>			
40J(R)	-.029	.015	NS
40J(L)	-.028	.008	NS
79J(R)	-.021	.003	NS
79J(L)	-.021	.009	NS
78J(R)	-.030	.012	NS
			$F_{50}^4 = 1.0153(NS)$
<u>6 x 10⁵ iu through conjunctiva</u>			
40J(R)	-.040	.017	NS
36J(L)	-.048	.012	NS
79J(L)	-.058	.031	NS
78J(R)	-.043	.004	NS
78J(L)	-.039	.009	NS
			$F_{35}^4 = 0.7359(NS)$
<u>3 x 10⁵ iu through conjunctiva</u>			
40J(L)	-.073	.037	NS
79J(R)	-.045	.013	NS
79J(L)	-.041	.009	NS
78J(R)	-.050	.019	NS
78J(L)	-.051	.020	NS
			$F_{17}^4 = 0.4043(NS)$

APPENDIX 7.1

THE CORRELATION BETWEEN PENICILLIN CONCENTRATION IN CONJUNCTIVAL SAC FLUID WITH TIME, AFTER TOPICAL TREATMENT.

Treatment/ animals/eyes	Coefficient of correlation	F ratio	df	Significance
<u>sodium benzyl penicillin</u>				
/5,000 iu/water				
94F(L)	-.9381	22.01	1:2	*
36J(R)	-.9618	61.69	1:4	**
76F(R)	-.9400	37.94	1:4	**
58F(L)	-.9937	316.57	1:3	**
40J(R)	-.9998	8265.82	1:2	**
<u>sodium benzyl penicillin</u>				
/5,000 iu/oointment				
58F(R)	-.9503	37.25	1:3	**
40J(R)	-.9504	65.30	1:6	**
94F(L)	-.9152	36.11	1:6	**
36J(L)	-.9335	20.32	1:2	*
76F(R)	-.8995	21.21	1:4	*
<u>procaine penicillin</u>				
/5,000 iu/oointment				
94F(L)	-.9853	100.00	1:2	**
58F(R)	-.9838	120.78	1:3	**
36J(R)	-.9919	183.32	1:2	**
40J(R)	-.8764	23.19	1:6	**
76F(L)	-.8785	20.30	1:5	**
<u>benethamine penicillin</u>				
/5,000 iu/oointment				
94F(R)	-.9710	49.52	1:2	*
58F(L)	-.9548	72.30	1:6	**
40J(R)	-.9868	185.02	1:4	**
76F(L)	-.9409	62.92	1:6	**

APPENDIX 7.2

HOMOGENEITY OF REGRESSION LINES DERIVED FROM DIFFERENT EYES
WHICH RECEIVED SAME TOPICAL INSTILLATIONS OF 5,000 iu PENICILLIN

Treatment/ animals/eyes	Regression coefficient	95% confidence limits: tSb	Significance of difference
<u>Sodium benzyl penicillin</u>			
0.1ml water			
94F(L)	-.2151	.1975	NS
36J(R)	-.1744	.0616	NS
76F(R)	-.1087	.0489	NS
58F(L)	-.1809	.0325	NS
40J(R)	-.1529	.0073	NS
			$F_{15}^4 = 2.5096 (NS)$
<u>Sodium benzyl penicillin</u>			
0.03gm ointment			
58F(R)	-.0458	.0238	NS
40J(R)	-.0454	.0137	NS
94F(L)	-.0549	.0223	NS
36J(L)	-.0442	.0422	NS
76F(R)	-.0354	.0214	NS
			$F_{21}^4 = .8216 (NS)$
<u>Procaine penicillin</u>			
0.03gm ointment			
94F(L)	-.0715	.0310	NS
58F(R)	-.0591	.0172	NS
36J(R)	-.0769	.0244	NS
40J(R)	-.0423	.0215	NS
76F(L)	-.0433	.0604	NS
			$F_{18}^4 = 1.411 (NS)$
<u>Benethamine penicillin</u>			
0.03gm ointment			
94F(R)	-.0434	.0196	NS
58F(L)	-.0270	.0075	NS
40J(R)	-.0403	.0076	NS
76F(L)	-.0342	.0099	NS
			$F_{18}^3 = 1.7924 (NS)$