STUDIES ON THE PHARMACOKINETICS AND SOME POTENTIAL ADVERSE EFFECTS OF TETRACYCLINES

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A thesis submitted in fulfilment for the degree of Doctor of Philosophy in the University of Nairobi.

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1985

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as University supervisors

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ACKNOWLEDGEMENTS

I am very grateful to my supervisors, Professors P. Løkken and K. Lindqvist for their guidance, criticism and useful suggestions. I am also grateful to Professor I. Nafstad who has been of constant academic help in this project. My thanks are also due to Professor A. Frøslie for his valuable help in the early stages of this project.

I wish to thank Professor D. Ngugi, Mr. J. Mbuvi, Mr. R. Kusewa and the Field Station staff, Faculty of Agriculture, for kindly providing some of the animals and for their assistance during this research. I am particularly grateful to Mrs. S. Sohlberg for her excellent and valuable technical assistance during my six months' research period in Norway and later in Nairobi.

The useful advice from Professor M. Yndestad and Dr. B. Nicolaissen is appreciated. Further, the technical assistance of I. Catrinius, H. Gildvedt and of the technical staff of the Department of Pharmacology and Toxicology, Veterinary College of Norway, is gratefully acknowledged.

The oxytetracycline preparations were generously

supplied by the "Pfizer Laboratory Ltd.", Nairobi, "A/S Apothekernes Laboratorium", Norway and "Rosco A/S", Denmark.

My thanks also go to Dr. J.M. Gathuma, Chairman of Department of Public Health, Pharmacology and Toxicology, University of Nairobi, for allowing me to carry out the research in this Department.

I gratefully express my appreciation of the role played by my husband, Dr. H.K. Maritim, on this project. His moral encouragement, participation in providing transportation at night, keeping us company at night during sample analysis, helping with statistical data analysis and taking care of the children in my absence is gratefully acknowledged.

I would also like to thank Mrs. W. Andreassen for typing the initial draft and Mr. J.K. Mbugua, for typing the final draft.

I wish to record my appreciation and gratitude to NORAD for the scholarship which enabled me to pursue these studies, and for the generous financial support of my work by a NORAD research/supporting grant, without which these studies would not have been possible.

A B S T R A C T

STUDIES ON THE PHARMACOKINETICS AND SOME POTENTIAL ADVERSE EFFECTS OF TETRACYCLINES

The tetracyclines have, next to the penicillins, been the most widely used antibiotics. There are still, however, problems and less well investigated aspects associated with their use.

The present investigation compares the pharmacokinetics and some postulated advantages of more recently introduced oxytetracycline (OTC) preparations with a conventional OTC product. The suitability of deep dewlap injection of OTC as an alternative route to intramuscular (IM) injection of the drug was also examined. A further aim has been to examine some potential adverse effects, especially a recent claim that tetracycline (TC) deposits in the eye may cause corneal discolouration and lens opacities in new-born animals if administered during pregnancy.

The pharmacokinetic studies required adequate methods for the determination of OTC levels in biological specimens. A microbiological agar diffusion method, with *Staphylococcus aureus* NCTC 6571 as test organism, proved to give reproducible and satisfactory results in preliminary experiments with rats and rabbits. The

lowest detectable concentrations for OTC were 0.1 µg/ml serum and 0.2 µg/g tissue. While the addition of various cations reduced the OTC activity in the test system, the sensitivity of the system was increased when EDTA was added in a concentration between 0.12 and 1 mmol. Comparison of the microbiological assay with high pressure liquid chromatography (HPLC) gave similar results and sensitivities with respect to OTC in plasma and tissue samples, while the determination of OTC in ruminal fluid was only possible with the microbiological assay, since the fluid contained substances which interferred with the HPLC determination. Autoradiography with ^{3}H-labelled TC was also introduced to trace the distribution and accumulation of the drug in various tissues.

A cross-over trial on 8 calves was conducted to compare two OTC preparations, a "long-acting", OTC-LA (Terramycin^{R}/LA long acting injectable solution, "Pfizer") and a "conventional" formulation, OTC-C (Terramycin^{R}-100 injectable solution, "Pfizer"), with regard to the serum levels after IM injection and after injection in the dewlap, as well as the extent and nature of local tissue damage and the drug residue concentrations at the sites of injection. After IM injection of the same dose of OTC-C as recommended for OTC-LA (20 mg OTC/kg bwt), the resulting serum concentrations were not significantly different. Maximum values were reached

after 4 hours and averaged 6.7 ± 2.2 µg OTC/ml after OTC-C, compared with 7.5 ± 2.5 µg OTC/ml after OTC-LA administration. After injection in the dewlap, the serum concentrations were also similar for the two preparations, but reaching peak concentrations of only 1.8 ± 0.2 and 1.7 ± 0.3 µg OTC/ml 12 hours after injections of OTC-C and OTC-LA respectively. The therapeutic minimum concentration of OTC was assumed to be 0.5 µg OTC/ml. Following IM and dewlap injection of OTC-C, mean levels above this concentration were maintained for 45 and 57 hours respectively, while the corresponding values after OTC-LA were 52 and 62 hours. Only a very limited retard effect of OTC-LA could be demonstrated, and the study does not support the claim of the manufacturers that therapeutic serum concentrations are maintained for 3 to 5 days after a single injection of OTC-LA. At post mortem examinations performed 44 and 63 days after the IM administration, no macroscopic changes were detected after any of the two preparations, and neither UV-illumination, the microbiological assay, or the HPLC analyses revealed any residue of OTC at the IM injection sites. In contrast, post mortem examination 30 and 49 days after injection of OTC-LA in the dewlap, revealed pronounced tissue damage. When illuminated by UV-light, intense fluorescence indicated the presence of OTC. This was confirmed by both HPLC analysis and

the microbiological assay, which revealed that 2.1 and 1.6 mg OTC remained at the site of injection 30 and 49 days following administration of OTC-LA. After injection of OTC-C in the dewlap, there was far less tissue damage, and the OTC residues after 30 and 49 days were only 0.3 and 0.1 mg respectively.

According to these results, OTC-LA does not seem to offer significant advantages over OTC-C. The dewlap has been suggested as an alternative injection site, which compared to the IM route offers advantages from a residue point of view. It proved, however that after dewlap injection the area under OTC serum concentration-time curve was only about 56% of that after IM injection. The local tissue reaction, especially after dewlap injection of OTC-LA, also indicates that this route is not recommendable for the administration of tetracyclines The dewlap might, however, be a suitable site for testing tissue reactions caused by various drug formulations.

Aquacycline^{R} "Rosco" (OTC-A), a recently introduced aqueous OTC-formulation, has been claimed to render several advantages including low tissue irritation at the site of injection. In an experiment with 12 calves, Aquacycline^{R} in a 5% (OTC-A5) and a 10% (OTC-A10) solution, was compared with a "conventional" 10% OTC-preparation, OTC-C (Terramycin^{R}-100 injectable

solution, "Pfizer"), by injecting 20 mg OTC/kg bwt of these preparations in the dewlap and thereafter monitoring serum concentrations as well as tissue reactions and residues at the site of injection. OTC serum levels above 0.5 µg/ml were maintained for about 60 hours after all three preprations. During this period, however, OTC-A5 and OTC-A10 resulted in higher initial blood levels, reflected in 39 and 20% larger areas under the serum concentration-time curves as compared to OTC-C. The OTC-A preparations caused less swelling at the site of injection. A tendency towards less pronounced tissue reaction after OTC-A, was also observed at post mortem examinations 28 and 42 days after injection. The OTC residues at the injection site were smaller, after OTC-A5, but none of the preparations resulted in OTC-residues exceeding 0.3 mg. Accordingly the present investigation gives support to the claims that OTC-A offers advantages with regard to absorption characteristics and tissue tolerance.

Gastrointestinal disturbances may accompany TC therapy, but only few studies have been performed concerning its effect on ruminal fermentative activity. A study of this, which also involved the examination of transfer of OTC from blood to the rumen and vice versa after intravenous (IV) (10 and 20 mg OTC/kg bwt) and intraruminal(IR) (3 mg OTC/kg bwt) administration,

was carried out in sheep. Following IV injection, OTC could not be detected in the ruminal fluid, and neither was it possible to detect OTC in plasma after IR administration. The production in the rumen of acetic, propionic and butyric acids was chosen as an indicator for the effect of OTC on the fermentative activity of the ruminal microflora. Volatile fatty acid determinations were carried out using gas-liquid chromatography. Although the level of OTC remained above 0.5 µg OTC/ml for about 40 hours after IR administration, there was no significant inhibition of the total volatile acid production.

Development of corneal discolouration and lens opacities in new-born rats due to systemic use of TC in pregnancy was recently reported, Krejči *et al.*, *Ophthalmic Res. 12:* 73 - 77, 1980. It was decided to undertake follow-up studies on this alarming and so far unsubstantiated adverse drug effect. In the present studies the potential of TC to be deposited and induce changes in the foetal eye was investigated by the administration of isotope-labelled and unlabelled TC to pregnant rats and rabbits. Administration of 20 mg TC/kg bwt/day from day 13 or 12 of gestation till term, had no effect on the length of pregnancy, litter size or gross morphology of the offspring. Examination by fluorescence microscopy of cryostat and paraffin-embedded

sections of eyes from the offspring, did not reveal TC deposits in the cornea or lenses of either rats or rabbits, and no morphological changes were seen in the stained paraffin sections. The possibility of TC deposition in the foetal eye was also examined with whole-body autoradiography after IV injection of ^{3}H-labelled TC in rats on day 9 and 20 and in rabbits on day 12 and 28 of gestation. The animals were killed at various intervals after administration and the pregnant uteri and the maternal kidneys and livers were examined by autoradiographic procedures. Twenty minutes after administration, ^{3}H-activity had accumulated in the placenta, uterine wall, maternal kidney and liver. A low level of activity was present in the foetal skeleton, while no radioactivity could be detected in the foetal lenses or the brain. When a longer time had elapsed, the radioactivity in the foetal/maternal tissues had decreased, and still no radioactivity was detectable in the foetal eye. Accordingly, the results of the present investigations do not support the recent report that the administration of TC to pregnant animals results in the deposition of TC in the foetal eye causing corneal discolouration and lens opacities.

CHAPTER ONE

INTRODUCTION

The tetracyclines are broad spectrum antibiotics which have been widely used in human and veterinary medicine for more than three decades. There are still, however, less investigated aspects of their pharmacology and toxicology.

The main objectives of this thesis were:

- to study the pharmacokinetics and examine some postulated advantages of two recently introduced oxytetracycline preparations in clinical comparative trials with a conventional oxytetracycline product,
- to examine the suitability of deep dewlap injection of oxytetracycline as an alternative to intramuscular injection,
- to study the passage of oxytetracycline across the ruminal wall, and its effect on ruminal fermentation,
- to examine whether tetracycline may cause corneal discolouration and lens opacities in new-born animals if administered during pregnancy.

Increasing attention has been paid to problems connected with residues and tissue damage at intramuscular (IM) injection sites (Rasmussen and Høgh, 1971; Rasmussen, 1979). The dewlap has been suggested as an alternative injection site (Bergsjø, 1976). It is a skin fold of no economic value which can easily be removed during meat inspection. Another possible advantage could be slower absorption resulting in prolonged action of the drug.

The study reported in *Chapter 4* was designed as a cross-over trial with IM and dewlap injections of oxytetracycline (OTC) in calves, aimed at comparing OTC serum concentrations as well as residue levels and tissue damage at the injection sites. Both a "conventional" (Terramycin^{R}-100, "Pfizer", USA) and a "long-acting" (Terramycin^{R}/LA, "Pfizer", USA) OTC preparation were injected. By giving a controlled precipitation of OTC at the injection site without significant tissue damage, the latter preparation is claimed to maintain therapeutic serum concentrations for 3 to 5 days, thereby reducing the cost and the need for repeated injections (Simpson, 1978; Cornwell, 1980). In addition to the comparison of the two routes of administration, this trial aimed at examining the postulated advantages of the long-acting preparation by comparing it with the conventional formulation.

Aquacycline^{R} ("Rosco, A/S", Denmark) is another OTC formulation which has recently been marketed with the claim that it offers absorption characteristics which maximise the antibacterial activity, besides being advantageous both with regard to local and systemic toxicity. The study presented in *Chapter 5* was aimed at examining how favourable some of the properties of this preparation actually are compared with a "conventional" OTC preparation (Terramycin^{R}-100, "Pfizer", USA). This was done by comparison of the two preparations in calves with respect to OTC bioavailability and serum concentrations, as well as local tissue reactions and residues following dewlap injection.

There are many reports on gastrointestinal disturbances associated with tetracycline therapy. Only few studies, however, have dealt with their effect on ruminal fermentative activity (Behravesh *et al.*, 1982; Jenkins, 1982). An important part of the fermentative activity of the ruminal microorganisms is the production of volatile fatty acids (VFAs) which contribute upto 70% of the daily energy need of the animal. One objective of the investigation with sheep, reported in *Chapter 6*, was to examine the influence of OTC administered parenterally or intraruminally on the production of VFAs. Another objective was to study the extent of absorption of OTC from the rumen, as well as its excretion into the rumen following parenteral administration.

The studies in *Chapter 7* were initiated by an alarming and so far unsubstantiated report of eye changes with corneal discolouration and lens opacities in newborn rats due to systemic use of tetracycline in pregnancy (Krejči *et al.*, 1980). Krejči and co-workers checked the eyes on a slit lamp and examined them histologically in UV-light for tetracycline fluorescence. These methods were also used in the present study, but in addition to rats, rabbits were also used, and methods for detection of tetracycline deposits were supplemented by autoradiographic studies with tritium-labelled tetracycline.

In order to carry out these studies, adequate methods had to be selected, and their performance in our hands evaluated. *Chapter 3* is a methodological section which includes studies on the applicability of the following methods for detection and quantitation of tetracyclines: a microbiological agar diffusion method, a high pressure liquid chromatography method, a fluorescence technique and a whole-body autoradiography technique.

CHAPTER TWO

LITERATURE REVIEW

2.1 TETRACYCLINES - GENERAL INTRODUCTION

The discovery of the tetracyclines was the result of a systematic screening of soil specimens collected from many parts of the world for antibiotic-producing micro-organisms. The first tetracycline to be introduced was chlortetracycline in 1948, followed closely by oxytetracycline and then tetracycline, a reduction product of chlortetracycline. In 1957, demeclocycline was developed and became available for general use in 1959. These four tetracyclines are all natural products that have been isolated from species of *Streptomyces*. Subsequently, several semisynthetic derivatives have been introduced.

The generic term tetracycline is used to describe the whole group, but it is also the name of a specific compound. Although there are specific and useful differences between the tetracyclines, they are in the main very much alike.

Unlike the penicillins and the aminoglycosides they are bacteriostatic at the concentrations usually achieved in the body but act similarly to the amino-

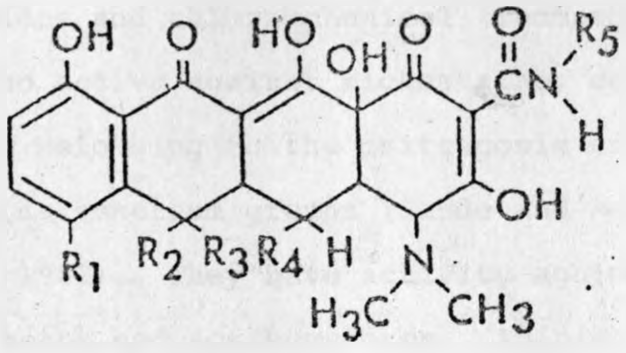
glycosides by interfering with protein synthesis in susceptible microorganisms. They all have a broad spectrum of antimicrobial activity against gram-positive and gram-negative bacteria, chlamydiae, rickettsiae and mycoplasmata, but the emergence of resistant strains and the development of other anti-microbial agents have reduced their value. Adverse effects have also restricted their usefulness.

2.2 PHYSICAL AND CHEMICAL PROPERTIES

The tetracyclines are closely congeneric derivatives of the polycyclic naphthacene-carboxamide. Their structural formulae are shown in Table 1, p.7.

The crystalline tetracycline bases are yellowish, odourless, slightly bitter and highly stable. They are only slightly soluble in water at a pH 7 (0.25 to 0.5 mg/ml), but readily form soluble sodium and hydrochloride salts. Aqueous solutions usually show appreciable loss of activity within 24-48 hours, particularly at an elevated pH (Thompson, 1976). Oxytetracycline dissolved in a propylene glycol-water solution has been found to be stable for relatively long periods (Huber, 1982). Stable chelate complexes are formed with metals such as calcium, magnesium, iron and aluminium.

Table 1 The years of introduction and the structural formulae of some tetracyclines (Modified from Thompson, 1976)



Drug	Year introduced	Substitutions				
		R_1	R_2	R_3	R_4	R_5
Chlortetracycline	1948	Cl	CH_3	OH	H	H
Oxytetracycline	1950	H	CH_3	OH	OH	H
Tetracycline	1952	H	CH_3	OH	H	H
Demeclocycline	1959	Cl	H	OH	H	H
Methacycline	1961	H	CH_3	H	OH	H
Doxycycline	1966	H	CH_3	H	OH	H
Minocycline	1970	$N(CH_3)_2$	H	H	H	H

2.3 ANTIMICROBIAL SPECTRUM AND RESISTANCE

The tetracyclines possess a wide range of antimicrobial activity which overlaps that of many other antibiotics, such as the penicillins and cephalosporins, the aminoglycosides and chloramphenical (Thompson, 1976). They are also active against rickettsiae, certain large viruses belonging to the psittacosis and the lymphogranuloma venereum groups (Sande and Mandell, 1980; Huber, 1982). They have activity against mycoplasma, spirochetes and actinomycetes. In high doses, some antiprotozoal activity has been observed (Huber, 1982). The tetracyclines are not active against any of the true viruses, yeasts or fungi.

Resistance to the tetracyclines develops relatively slowly in susceptible organisms. Resistant strains of staphylococci, coliform bacilli, pneumococci and haemolytic streptococci are common. Resistant strains of *Haemophilus influenzae* and *Clostridium welchii* have also been reported (Weinstein, 1975; Sande and Mandell, 1980; Huber, 1982). Microorganisms that have become insensitive to one tetracycline frequently exhibit resistance to the others. Resistance to the tetracyclines in *E. coli* and probably in other bacterial species is mediated by a plasmid. Plasmids that impart resistance contain genetic information for a number of

proteins that appear to affect transport of the drug into the cell (Sande and Mandell, 1980). It has been suggested that ethylenediamine tetraacetate might bind divalent ions in the cell wall thereby increasing the permeability of resistant organisms to tetracyclines and other agents (Weiser *et al.*, 1968; Wooley *et al.*, 1981; 1982).

2.4 ROUTES OF ADMINISTRATION

The tetracyclines are available in a wide variety of forms for oral, topical and parenteral administration. In veterinary medicine they are often administered by the intravenous (IV) or the intramuscular (IM) routes. When given parenterally to large animals, the dosage is commonly within the range of 4.4-11 mg/kg bwt daily. Orally, tetracyclines are administered to small animals in a dose range of 33-110 mg/kg/day, preferably divided into 2 or 3 doses (Huber, 1982). Except for local ophthalmic use and intramammary infusion for the treatment of mastitis, topical use of the tetracyclines is best avoided because of the resistance risk (Huber, 1982).

2.5 PHARMACOKINETICS

2.5.1 Absorption

Most of the tetracyclines are adequately, but incompletely absorbed from the gastrointestinal tract. The absorption

takes place mainly from the stomach and the first part of the intestine. The percentage of an oral dose that is absorbed on an empty stomach in humans, has been found to be lowest for chlortetracycline (30%); intermediate for oxytetracycline, demeclocycline and tetracycline (60% to 80%); and high for doxycycline (95%) and minocycline (100%) (Barza and Scheife, 1977).

Since the tetracyclines form non-absorbable chelates with heavy metals, absorption is also impaired to a variable degree by milk and milk products, and particularly by the concomitant administration of aluminium hydroxide gels, sodium bicarbonate, calcium and magnesium salts and iron preparations (Neuvonen, 1976).

Intramuscular injections of tetracycline result in detectable plasma levels after about 15 minutes and peak levels are reached within 1-2 hours. Plasma levels above 0.5 µg/ml are maintained for about 12 hours and decline to trace amounts within 24 hours after injection (Huber, 1982).

In human medicine, 0.5 - 1µg/ml has been accepted as adequate for most purposes in the treatment of adults. It has been assumed that this level is adequate in animals also, and this is borne out by clinical experience (Bywater, 1982).

2.5.2 Distribution

The volume of distribution of the tetracycline is larger than that of total body water, partly due to binding to plasma proteins. Approximate values for plasma protein binding are summarised in Table 2. The binding of oxytetracycline is relatively small compared with the other tetracyclines.

Table 2 Some pharmacokinetic characteristics of tetracyclines (Modified from Thompson, 1976).

Drug	Plasma protein binding, %	Plasma half-lives, hours	Renal clearance, ml/min
Chlortetracycline	40-70	4-6	30
Oxytetracycline	20-35	8-10	85
Tetracycline	25-60	8-9	60
Demeclocycline	40-90	10-17	25-35
Methacycline	75-90	10-16	30
Doxycycline	25-90	12-20	20-30
Minocycline	70-75	12-19	10

All the tetracyclines are concentrated by the liver and then excreted by way of bile, into the intestine from which they are partially reabsorbed. Biliary concentrations of these agents are at least five to

ten times higher than the simultaneous values in plasma (Acocella *et al.*, 1968).

Tetracycline levels in the cerebrospinal fluid are primarily dependent on the route and duration of therapy rather than on actual dosages and usually average about one fifth of simultaneous plasma levels (Thompson, 1976). The IV injection of a tetracycline results in a gradual appearance of the drug in the spinal fluid over a period of 6 hours. Oral therapy yields very low spinal fluid concentrations. A higher ratio of cerebrospinal fluid to plasma concentrations has been reported with minocycline compared with doxycycline.

Penetration into most other fluids and tissues is excellent. Concentrations in synovial fluid and the mucosa of the maxillary sinus approach that of plasma (Parker and Schmid, 1971; Lundberg *et al.*, 1974). Minocycline is more lipophilic than doxycycline, oxytetracycline or tetracycline; and reaches a sufficient concentration in tears and saliva to eliminate meningococcal carrier state (Hoeprich and Warshauer, 1974). The tetracyclines accumulate in the reticuloendothelial cells of the liver, spleen and bone marrow, and at the sites of new bone formation and in enamel and dentine of teeth under formation. They also tend to accumulate in inflammatory tissue, and cling to some neoplastic tissues for surprisingly long time (Rall *et al.*, 1957).

The tetracyclines cross the placenta and enter the foetal circulation and the amniotic fluid, reaching about half of the concentration in the maternal blood (Huber, 1982).

2.5.3 Excretion

All the tetracyclines are excreted in urine and faeces, with glomerular renal excretion as the primary route for most. Values for plasma half-lives and renal clearance of tetracyclines are presented in Table 2, p. 11.

Chlortetracycline is more dependent on biliary excretion for its elimination in contrast to the other tetracyclines. Doxycycline is excreted in the faeces largely as an inactive conjugate or as a chelate and has less impact on normal microflora (Hinton, 1970; Barlett *et al.*, 1975).

In the milk, tetracyclines are found in a concentration of about one-half of that in the maternal serum (Huber, 1982).

2.6 PHARMACODYNAMICS

Results of many studies have supported the early suggestion that inhibition of protein synthesis is responsible for tetracycline-induced bacteriostasis (Gale and Folkes, 1953). The site of action of

tetracyclines is the bacterial ribosome, but at least two processes appear to be required for these antibiotics to gain access to the ribosomes of gram-negative bacteria. The first is passive diffusion through hydrophilic pores in the outer cell membrane. The second process involves an energy-dependent active transport system that transfers all tetracyclines through the inner cytoplasmic membrane. Such transport may require a periplasmic protein carrier (Franklin and Higginson, 1970). The permeation of the tetracyclines into gram-positive bacteria is less well understood, but also requires an energy-dependant sysytem (Sande and Mandell, 1980). The tetracyclines inhibit protein synthesis in bacteria by blocking the combination of aminoacyl-transfer RNA with the messenger RNA-ribosome complex (Suzuka *et al.*, 1966). There is a reversible binding to the 30S ribosomal sub-unit. In a sufficiently high concentration, the tetracyclines will also impair protein synthesis in mammalian cells, but the host cells lack the active transport system found in bacteria (Sande and Mandell, 1980).

2.7 UNTOWARD EFFECTS

2.7.1 Toxic effects

Tetracyclines are relatively non-toxic, but they are nevertheless liable to cause a number of untoward effects.

Gastrointestinal effects - The tetracyclines produce gastrointestinal irritation. This may vary with the compound and the dose, and is also subject to individual and species variation. In humans, effects such as epigastric burning, distress, abdominal discomfort, nausea and vomiting may occur, particularly after oral administration. Conventional dosages of tetracycline and oxytetracycline given orally to cats or IV to horse, will often produce serious gastrointestinal disturbances. In cats, clinical signs may consist of diarrhoea, colic, emesis, depression, fever and anorexia (Wilkinson, 1968). In horses, similar signs, including apathy, severe diarrhoea and even death have been observed (Cook, 1973; Baker and Leyland, 1973). It is possible that a combination of gastrointestinal irritation and alteration of the intestinal flora is responsible for the high occurrence of gastrointestinal disturbances in cats and horses. In ruminants the main complication following oral therapy is alteration of the normal microflora, so that the animal becomes anorexic due to digestive disturbances frequently accompanied by diarrhoea (Bywater, 1982; Huber, 1982).

Hepatotoxicity - Liver damage due to tetracyclines was first reported by Lepper (1951) in patients receiving large doses. Oxytetracycline and tetracycline appear

to be less hepatotoxic than the other drugs of this group (Sande and Mandell, 1980). Pregnant women appear to be particularly susceptible to severe tetracycline-induced liver damage (Schultz *et al.*, 1963). The clinical signs and symptoms include jaundice, azotemia, acidosis and terminal shock. The livers are diffusely infiltrated with fat. Fatty changes in the liver resulting from parenteral administration of tetracyclines have also been described in experimental laboratory animals (Combes *et al.*, 1972; Gray *et al.*, 1974).

Nephrotoxicity - The tetracyclines, with the exception of doxycycline, are not recommended for administration to patients with impaired renal function. The untoward effects are directly related to the particular tetracycline used, the dose and the extent of renal disease; and they are probably related to the anti-anabolic effects of the tetracyclines (Shils, 1963). Among the toxic signs are progressive azotemia, acidosis, hyperphosphatemia, negative nitrogen balance, potassium loss in urine, anorexia, nausea and vomiting.

Bones and teeth - Tetracyclines are deposited in calcifying areas in bones and in mineralizing zones of the teeth. There is some evidence that the tetracyclines may interfere with the growth of the foetus and young infant (Cohlan *et al.*, 1963). The

tetracyclines may cause yellow or brown discolouration of the teeth and dental hypoplasia. There are differences in the degree of tooth change induced by various tetracyclines. As early as in 1965, oxytetracycline was found to stain teeth less than other tetracycline analogues (Weyman, 1965), and this has later been verified in several studies (Moffitt *et al.*, 1974). From animal experiments one would expect dental involvement to be less of a problem with the semi-synthetic derivative of oxytetracycline, doxycycline (Klingeren, 1977).

Miscellaneous effects - Intravenous administration of a tetracycline is frequently followed by thrombophlebitis, especially when a single vein is used for repeated infusion. The reaction can be minimized by keeping the tetracycline concentration less than 0.5% (Thompson, 1976). The irritative effects of these agents are evident by the pain experienced at the injection site and the tissue damage observed when injected IM (Rasmussen and Høgh, 1971; Immelman *et al.*, 1978).

Rapid IV injection of OTC in cattle can produce acute collapse (Mathew *et al.*, 1978; Gross *et al.*, 1979). In horses it is characterised by shivering, ataxia, dypsnoea and collapse (Potter, 1973). The mechanism for the depression of cardiovascular and neuromuscular

functions may be related to their ability to chelate divalent ions, especially calcium (Cohen *et al.*, 1970; Gyrd-Hansen *et al.*, 1981). Bradycardia associated with the tetracyclines has also been described in guinea pigs (Armah, 1974) and rabbits (Tauberger *et al.*, 1971).

Long-term therapy with the tetracyclines may produce changes in the peripheral blood, such as leukocytosis, atypical lymphocytes and thrombocytopenic purpura.

The tetracyclines may increase the intracranial pressure and cause tense bulging of the fontanels in young infants, even when given in therapeutic doses. Discontinuation of therapy results in prompt return of the pressure to normal.

That the tetracyclines might be responsible for congenital cataracts was first suspected by Harley *et al.*, 1964; 1965. Eye changes due to the use of systemic tetracycline have been reported in new born rats (Krejci *et al.*, 1980).

2.7.2 Hypersensitivity reactions

Allergy due to tetracyclines has been reported in humans but is rare. Various skin reactions have included morbilliform rashes, urticaria and generalised

exfoliative dermatitis. Other reported reactions are burning of the eyes, cheilosis, atrophic or hypertrophic glossitis, pruritus ani or vulvae vaginitis.

2.7.3 *Biological effects*

Tetracyclines are true broad-spectrum antibiotics and for that reason they are probably overused. Such excessive use has led to the development of many resistant strains of bacteria and to superinfections. Among the most important superinfections are those that involve the gastrointestinal tract. The tetracyclines depress the normal microflora and after a couple of days of therapy, the faeces become soft, unformed and odourless. Superinfecting organisms are usually antibiotic resistant staphylococci, various strains of *Proteus*, and *Candida albicans*.

2.8 THERAPEUTIC AND OTHER USES

The tetracyclines have been used extensively for the treatment of infectious diseases and previously also as additives to animal feeds to promote growth.

Important diseases and aetiological agents which have been commonly treated with the tetracyclines are presented in Table 3, pp. 21-22. Because of the emergence of resistant organisms and the discovery of agents with narrower antimicrobial spectra, tetracyclines are not

generally the antibiotics of choice in gram-positive or gram-negative infections. However, they have a place in the treatment of chlamydial, rickettsial and mycoplasmal infections, and also in acute exacerbations of chronic bronchitis, in non-specific urethritis, brucellosis, plague and cholera; low doses are used in the long-term treatment of severe acne.

Discoveries by Japanese workers of transmissible antibiotic resistance suggested that tetracycline-resistant *E. coli* from animals fed tetracyclines might constitute a reservoir of resistance potentially transmissible to pathogenic *E. coli* and *Salmonella* affecting man (Hudd, 1983). Therefore, in the European Economic Community and many other countries, antibiotics such as tetracyclines and penicillin have been banned as growth promoting additives in feeds.

Other uses of tetracyclines include diagnostic tests. The tetracyclines become attached to certain neoplastic tissues for a surprisingly long time and exhibit a golden yellow fluorescence when exposed to UV-light. They have also been used as a marker of bone growth (Cohlan *et al.*, 1963)

Table 3 Diseases treated with tetracycline antibiotics (From Huber, 1982)

Aetiological agent	Disease
Actinobacillosis lignieresi	Actinobacillosis
Actinomyces bovis	Actinomycosis
Aerobacter aerogenes	Mastitis
Anaplasma marginale	Anaplasmosis
Bacillus antracis	Anthrax
Borrelia anserina	Avian borreliosis
Brucellas canis	Canine brucellosis
Clostridium chauvoei	Blackleg
C. hemolyticum	Bacillary haemoglobinuria
C. novyi	Infectious necrotic hepatitis
C. perfringens B, C, D	Enterotoxemia
C. septicum	Malignant oedema
C. tetani	Tetanus
Corybebacterium equi	Foal pneumonia
C. pyogenes	Mastitis
C. renale	Bovine pyelonephritis
Cowdria ruminantium	Heartwater disease
Dermatophilus congolensis	Cutaneous streptothricosis
Erysipelothrix insidiosa	Erysipelas
Escherichia coli	Mastitis, colibacillosis
Fusiformis necrophorus	Oral and hepatic necrobacilloses, infectious pododermatitis
Haemobartonella canis	Canine bartonellosis (tetracycline used concurrently with oxophenarsine)

Table 3 (contd.) Diseases treated with tetracycline antibiotics (From Huber, 1982)

Aetiological agent	Disease
Hemophilus spp.	Respiratory infections
H. suis	Infectious polyarthritis
Leptospira spp.	Leptospirosis
Listeria monocytogenes	Listeriosis
Moraxella bovis	Bovine infectious keratitis
Mycoplasma spp.	Mastitis, serositisarthritis, agalactia
M. hyponeumoniae	Porcine enzootic pneumonia
Nanophyetus salmincola	Canine rickettsiosis
Pasteurella anatipestifier	Pasteurellosis in pheasants
P. hemolytica	Mastitis, pasteurellosis
P. multocida	Pasteurellosis, fowl cholera, haemorrhagic septicemia
Salmonella abortus-ovis	Abortion
Shigella equirulis	Shigellossis of foals
Staphylococcus aureus	Mastitis, synovitis
S. hyicus	Exudative epidermitis
S. hyos	Exudative epidermitis
Streptococcus agalactiae	Mastitis
S. dysgalactiae	Mastitis
S. equi	Strangles
S. uberis	Mastitis
Vibrio fetus	Ovine vibriosis

2.9 RESIDUES IN EDIBLE TISSUES OF ANIMALS

The use of antibiotics in veterinary medicine should not leave unacceptable levels of residues in meat, milk or eggs destined for human consumption. The hazards may include hypersensitivity reactions and the development of antibiotic resistance in pathogenic organisms transimissible to man. The withholding times and tolerance levels of some tetracyclines are presented in Table 4, p. 24.

The use of doxycycline and minocycline in food-producing animals may be limited because of persistant drug residues (Aronson, 1980). Addition of tetracyclines to animal feeds at 5-20 ppm does not seem to result in detectable levels of residues in edible tissues.

2.10 ASSAY TECHNIQUES

Various techniques have been applied for the analysis of tetracyclines. For routine determinations in clinical practice, microbiological assays are commonly used. They are simple and inexpensive, but they do not allow differentiation between the tetracyclines themselves and active metabolites. Further, they do not respond to inactive products of degradation. These methods have also been used for the detection and quantitation of tetracycline residues in animal tissues and dairy products (Kirshbaum *et al.*, 1967; Kramer *et al.*, 1968) and in the assaying of tetracycline added to animal feed (Horwtzt, 1970).

Table 4 Withdrawal times and tolerance levels of some tetracyclines (Modified from Booth, 1982).

Drug	Species	Pre-slaughter withdrawal time, days	Tolerance levels, ppm
Oxytetracycline			
parenteral	cattle	22	0.1 (meat)
	pigs	26	0.1
oral	cattle	7	0.1
	pigs	26	0.1
Chlortetracycline			
oral	cattle	21	0.1
			0 (fat, milk)
	calves	3	4 (liver, kidney)
			1 (muscle, fat)
	pigs	5	1 (muscle)
			4 (kidney)
			2 (liver)
Tetracycline			
oral	calves	5	0.25 (meat)
	sheep	-	0.25
	pigs	4	0.25

Several chemical methods have also been developed for the analysis of the tetracyclines. Tetracyclines have been quantitated by fluorimetry after extraction of chelates formed with calcium, magnesium or aluminium ions (Kohn, 1961; Lever, 1972; Wilson *et al.*, 1972). Column chromatography (Fike and Brake, 1972), paper chromatography and thin layer chromatography (Wagman and Weinstein, 1973; Ryan and Dupont, 1974) have also served as separation techniques. More recently, separation has been performed with high pressure liquid chromatography usually followed by quantitation with spectrophotometer (Bøcker and Estler, 1979; Hermansson, 1982). The method is rapid and relatively accurate.

Tetracycline analyses have also been performed with the radioimmunoassay method. This method is sensitive and specific (Faraj and Ali, 1981). The whole-body autoradiography technique has been used for studying the general distribution patterns of radioisotope-labelled tetracyclines (Blomquist and Hanngren, 1966). The technique was first introduced by Ullberg and his associates in 1954, and has since then been further developed and improved.

The emitted fluorescence when tetracycline-containing tissues are exposed to UV-light will also demonstrate tetracycline localisation and allow semiquantitative assessments.

CHAPTER THREE

METHODS FOR DETECTION AND QUANTITATION OF TETRACYCLINES

3.1 INTRODUCTION

Several methods are available for quantitative or semiquantitative estimation of tetracyclines in biological specimens. The following methods were selected and their applicability evaluated for use in pharmacokinetic studies of tetracyclines: a microbiological agar diffusion method, a high pressure liquid chromatography method, a fluorescence technique and a whole-body autoradiography technique.

The microbiological agar diffusion method is based on the principle that the concentration of the antibiotic in the sample is exponentially proportional to the inhibition of bacterial growth (Grove and Randall, 1955; Kavanagh, 1963). This method allows quantitation of the antibiotic, but metabolites without antibacterial activity will not be detected. The agar diffusion method was first evaluated in pharmacokinetic studies with rabbits and rats. Further tests were done to examine the effect of some cations and ethylenediamine tetraacetate (EDTA) on the size of the oxytetracycline inhibition zones.

The high pressure liquid chromatography (HPLC) method used for the quantitation of oxytetracycline was

the same as that described by Hermansson (1982) for tetracycline analysis. After extraction of the tetracycline from biological specimens and separation by HPLC, it was monitored by an UV-spectrophotometer. This method allows analyses of the antibiotic as well as its degradation products from biological material (Bøcker and Estler, 1979a). The performance of the HPLC was compared to that of the agar diffusion method in parallel analyses of plasma samples containing oxytetracycline.

The fluorescence technique is based on the emission of golden-yellow light by the complex aromatic structure of the tetracycline molecule when exposed to UV-light. It has proved useful as a semiquantitative method in pharmacokinetic studies of tetracyclines (Helander and Bottiger, 1953; Bottiger, 1955).

The whole-body autoradiography (WBA) method requires an adequate isotope-labelled preparation of the drug. The technique enables a semiquantitative visualisation of the distribution and localisation of the drug and its isotope-labelled metabolites. Tritium-labelled tetracycline was selected and the method of Ullberg (1977) was used.

3.2 THE MICROBIOLOGICAL AGAR DIFFUSION METHOD

3.2.1 Materials and Methods

Culture media - Mueller Hinton broth (CM 405) and Mueller Hinton agar (CM 337) were prepared as recommended by the manufacturer ("Oxoid Ltd.," Basingstoke, Hampshire, England).

Test organism - A stock culture of *Staphylococcus aureus* (NCTC 6571) was prepared as follows: The organism was inoculated in Mueller Hinton broth and incubated for 14 hours at 37°C before streaking on blood agar and Mueller Hinton agar plates. Selected colonies from the plates were streaked heavily on several Mueller Hinton agar slants and incubated at 37°C for 24 hours. The growth was harvested from the slants by washing with 2-3 ml sterilized saline. The suspension was transferred to a Roux bottle containing 250 ml Mueller Hinton agar and distributed evenly over the surface of the agar with sterilized glass beads. The bottles were incubated at 37°C overnight. The resultant growth was washed with 100 ml of 12 mmol phosphate buffer, pH 7.0, and centrifuged. The supernatant was discarded and the cell deposit was further re-suspended in phosphate buffer containing 15% glycerine. To standardize the bacterial concentration, the turbidity of the suspension was adjusted with a Spectronic^{R} 20 photometer ("Bausch & Lomb", Rochester, NY, USA).

using buffer with glycerine, so that 0.1 ml of the suspension in 10 ml saline gave an optical density of 0.3 - 0.4 at 600 nm. The cell suspension was divided into 4 ml portions per Bijou bottle and kept frozen at -20°C. The optimal amount of inoculum proved to be approximately 1 ml cell suspension per 100 ml medium.

Oxytetracycline (OTC) - Oxytetracycline hydrochloride from "A/S Apothekernes Laboratorium", Oslo, Norway was used. A stock solution containing 100 µg OTC/ml was prepared by dissolving OTC in 0.01 N hydrochloric acid.

OTC Standards in buffer solution - The stock solution was diluted with 100 mmol phosphate buffer, pH 4.5, to give the following concentrations: 0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.0 µg OTC/ml.

OTC Standards in biological samples - Antibiotic-free serum, kidney, liver and muscle were obtained from control rats. OTC was added to the serum to obtain the following concentrations: 0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.0 µg OTC/ml. The tissues were cut into small pieces and homogenized in phosphate buffer and OTC was added to obtain the final concentrations of 0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.0 µg OTC/g of the initial amount of tissue.

Assays - Aliquots of molten Mueller Hinton agar cooled to 48°C were inoculated with the previously prepared stock culture (1 ml/100 ml medium). The agar was poured in 130 ml quantities onto framed glass plates (220 X 150 mm) and allowed to solidify on a levelled surface. The frame consisted of 4 pieces of glass suspenders 15 mm wide and 6 mm thick which were sealed to the plate with melted agar. Wells, 10 mm in diameter, were cut in the agar with a cork borer. Two hundred microlitre aliquots in triplicate of the prepared concentrations of OTC in buffer, serum and tissue samples, were pipetted in a random fashion into the wells by a micropipette (Cole-Parmer, Chicago, Illinois, USA). A pre-diffusion time at room temperature of one hour was used as recommended by Fabiansson and Rutegaard (1979), before incubation at 37°C for 18 hours. The diameters of the inhibition zones, including the 10 mm - diameter of the wells were measured with a vernier caliper to the nearest 0.5 mm. The measurements were averaged and plotted on the arithmetic scale of a semilogarithmic graph paper against the OTC concentrations on the logarithmic scale.

Calculations - The variations between the triplicates were calculated according to Yamane (1973).

For the standard curve, the best line of fit was

calculated by a curve-fitting programme (Hewlett-Packard HP-97 calculator) using the following equation: $Y = a + b \ln X$; where Y = inhibition zone (mm), a = Y-intercept, b = slope and X = OTC concentration (µg/ml or g).

3.2.2 Results and discussion

The standard curves with OTC dissolved in phosphate buffer were linear within the range of 0.05 - 1.0 µg OTC/ml (Fig 1, p. 32). The day to day variations between the standard curves were insignificant. The inhibition zones were not measureable below 0.05 µg OTC/ml, and this concentration therefore represented the lower sensitivity limit of the method.

Linear curves were obtained for OTC concentrations in biological specimens within the ranges of: 0.10 - 1.0 µg OTC/ml serum, 0.10 - 1.0 µg OTC/g kidney, 0.20 - 1.0 µg OTC/g liver and 0.10 - 1.0 µg OTC/g muscle (Fig. 2, p. 33), with the lower concentrations representing the lower sensitivity limits of the method.

The precision of microbiological agar diffusion method was very good, with coefficients of variation of less than 5% at all concentrations (Table 5, p. 34). Besides being sensitive and accurate, the microbiological agar diffusion method was found to be simple and inexpensive.

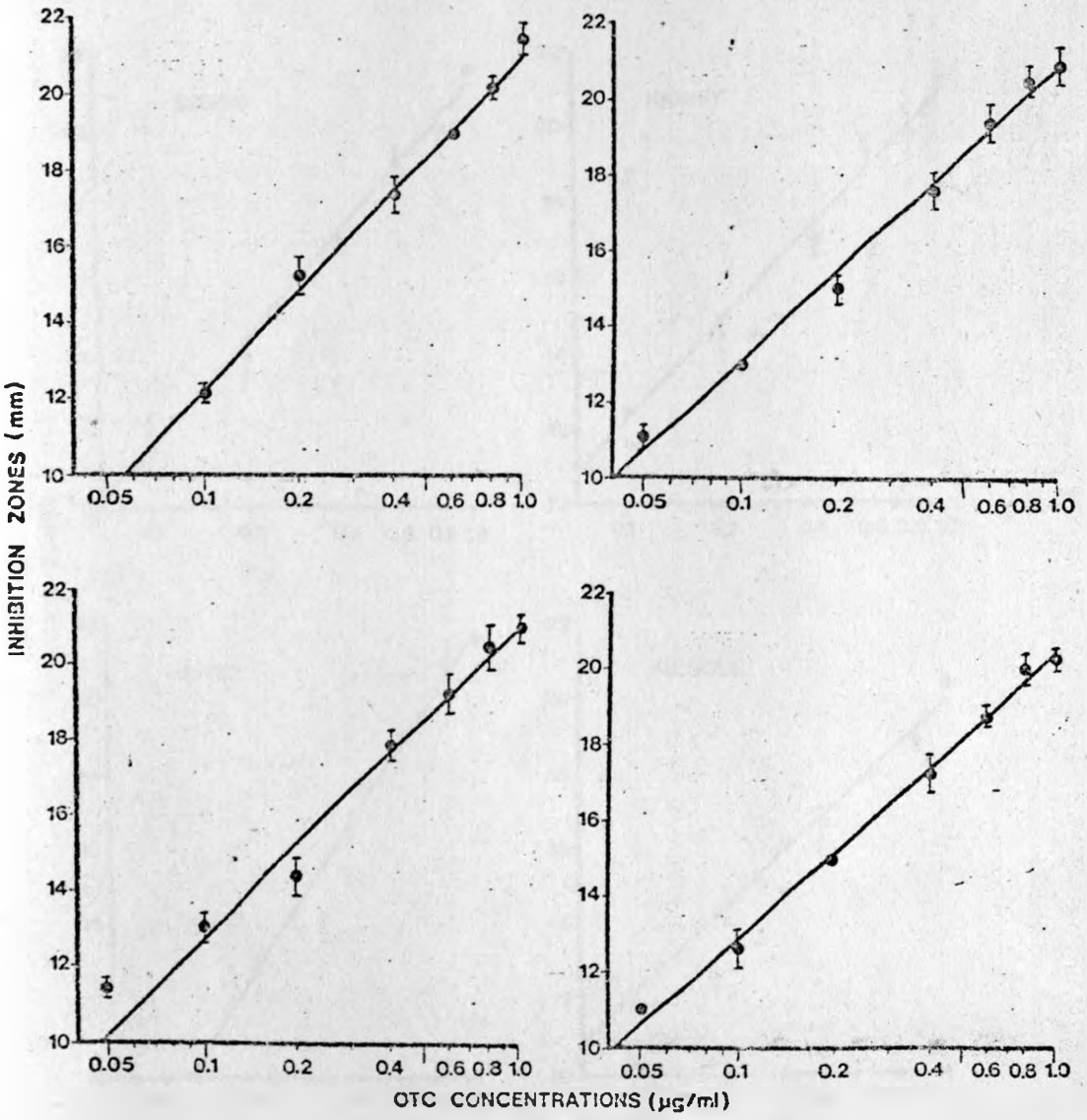


Fig. 1 Standard curves for oxytetracycline (OTC) concentrations in phosphate buffer, obtained by the microbiological agar diffusion method on different days (Diameter of the well = 10 mm)

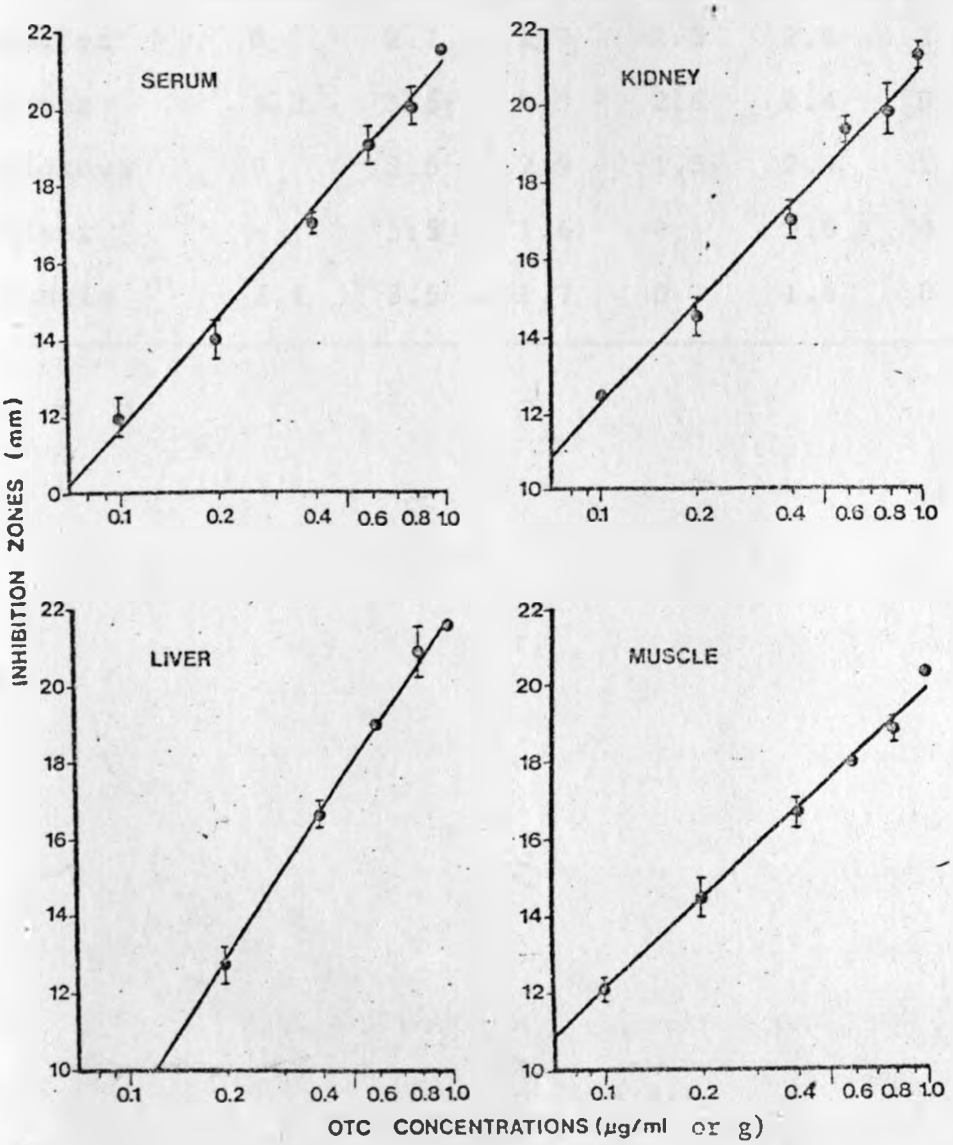


Fig. 2 Standard curves for oxytetracycline (OTC) concentrations in serum and tissues, obtained by the microbiological agar diffusion method. (Diameter of the well = 10 mm).

Table 5 Coefficients for the per cent variation of the OTC inhibition zones. Three determinations at each concentration in buffer, serum and tissues.

Medium or tissue	OTC Concentrations (µg/ml or g)					
	0.1	0.2	0.4	0.6	0.8	1.0
Buffer	0	2.7	2.7	2.5	2.0	2.3
Serum	4.2	3.6	0	2.6	2.4	0
Kidneys	0	3.5	2.9	1.5	2.9	1.4
Liver	-	3.5	1.6	0	2.8	0
Muscle	2.4	3.5	1.7	0	1.6	0

3.3. THE MICROBIOLOGICAL AGAR DIFFUSION METHOD TESTED IN PHARMACOKINETIC STUDIES WITH RABBITS AND RATS

3.3.1 Materials and Methods

Rabbits - Eight albino female rabbits (0.92 - 1.62 kg. bwt) were used. Half of them received 10 mg OTC/kg bwt by intramuscular (IM) injection, while the other half was given the same dose by slow intravenous (IV) injection (Terramycin^{R} - 100 injectable solution, 100 mg OTC/ml, "Pfizer", USA). Blood was drawn from the marginal ear vein before and after administration according to the following time schedules:-

IM: 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours

IV: 0, 5, 10, 15, 20, 30, 45 minutes; 1, 1.5, 2, 3, 4, 5 and 6 hours

The blood was allowed to clot before centrifugation and the sera obtained were kept at -20^{o}C until analysed.

Rats - Fourty albino female rats (190 - 240 g bwt) were used. Ten served as controls, while 30 received 60 mg OTC/kg bwt by IM injection in the hind thigh. At time intervals of 1, 2, 3, 4, 6 and 12 hours after drug administration, five rats were anesthetized with diethyl ether. Blood was then collected from the aorta and the sera obtained were stored as described for rabbits. The kidneys, livers and muscle from contralateral hind thigh of the injection site, were excised and kept at -20^{o}C.

Analyses - Determination of OTC in serum and tissues was done by the agar diffusion method (Ch. 3.2, p. 28). If necessary, the samples were diluted before assaying with control sera or control tissues to fall within the range of 0.1 - 1.0 µg OTC/ml or g tissue.

Calculations - Concentrations of OTC in serum and tissue samples were calculated from the standard curves (Ch. 3.2., p. 28).

3.3.2 Results and discussion

Rabbits - After IM injection the mean peak serum concentration, 3.3 ± 0.3 µg OTC/ml, was reached after 1 hour (Fig. 3, p. 38). The OTC levels remained above 0.5 µg OTC/ml for 6 hours. Five minutes after IV injection, an initially high mean serum concentration, 19.7 ± 3.3 µg OTC/ml was reached, the level declined rapidly but remained above 0.5 µg OTC/ml for 6 hours. Between the rabbits there were relatively small differences in the OTC serum concentrations both after IM and IV injections.

Rats - OTC levels in serum and tissues are summarised in Table 6, p.39. High concentrations, but with large variations were found in the kidney and liver tissues. The concentrations were maximal about 2 hours after IM administration.

These patterns of distribution and elimination of OTC agree well with the findings in other studies on rodents (Otte, 1960; Gray *et al.*, 1974; Bøcker and Estler, 1979a, b; 1981) and indicate that the method is suitable for investigations of the kinetic of oxytetracycline.

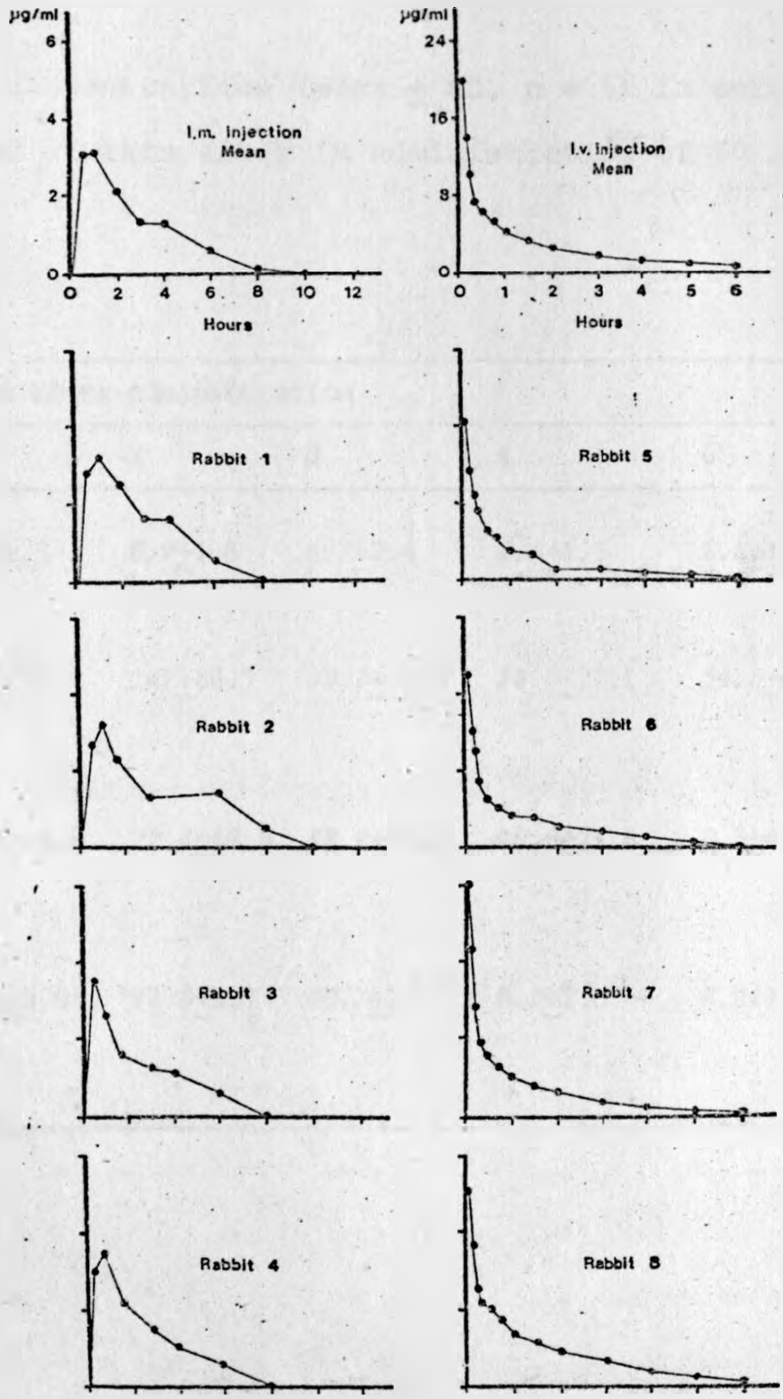


Fig. 3 Mean and individual OTC concentrations (µg/ml in serum of 8 rabbits following IM and IV injections of 10 mg OTC/kg bwt.

Table 6 OTC concentrations (*mean* ± SD, n = 5) in serum and tissue samples of rats after IM administration of 60 mg OTC/kg bwt.

Sample	Hours after administration					
	1	2	3	4	6	12
Serum (µg/ml)	*5.8*±1.5	*6.7*±1.6	*6.2*±2.4	*4.6*±1.6	*2.9*±1.9	*1.1*±0.4
Kidneys (µg/g)	*80.0*±59.9	*147*±66.7	*99.3*±32.9	*59.3*±28.1	*54.5*±23.6	*16.2*±6.0
Liver (µg/g)	*43.3*±29.8	*72.4*±48.8	*68.7*±43.5	*40.8*±21.6	*9.3*±4.2	*6.1*±2.1
Muscle (µg/g)	*10.7*±3.0	*14.9*±2.7	*10.3*±2.7	*8.9*±2.0	*8.2*±1.1	*3.8*±0.7

3.4 INFLUENCE OF SOME CATIONS AND EDTA ON OTC INHIBITION ZONES

3.4.1 Materials and Methods

Cations - 100 mmol stock solutions of Ca^{2+}, Mg^{2+} and Al^{3+} ions were prepared by dissolving calcium chloride, magnesium sulphate ("May and Baker Ltd.", Dagenham, England) and aluminium chloride ("Koch-Light Laboratory, Ltd.", Colnbrook, Bucks, England) in distilled water. The pH was adjusted to 7.2 - 7.4 with 10% sodium hydroxide solution before sterilization by membrane filtration using filters of pore size 0.45 µm. After dilution with pH adjusted sterile water, each cation solution was added to the wells or incorporated in the assay medium resulting in final concentrations of 0.05, 0.50 and 5.0 mmol, and the effects on the growth of the test organism and OTC inhibition zones were then examined.

EDTA - A 100 mmol EDTA stock solution was prepared by dissolving disodium ethylenediamine tetraacetate ("May and Baker Ltd.", Dagenham, England) in distilled water. The pH was adjusted to 7.2 - 7.4 with 10% sodium hydroxide and the solution was sterilized by membrane filtration. The effect of EDTA on the growth of the test organism and size of OTC inhibition zones

were examined as described for cations. Furthermore, the effects of EDTA on the test organism were examined by the turbidimetric assay method described by Dornbush and Abbey (1972).

3.4.2 Results and discussion

Cations alone - At concentrations below 5 mmol, none of the cations inhibited the growth of the test organism, neither when added to the wells nor when incorporated in the assay medium.

Cations and OTC - While Ca^{2+} ions had no prominent effects on OTC inhibition zones at concentrations of 0.05 and 5.0 mmol, there was a slight increase at 0.5 mmol (Fig. 4, p. 43).

Mg^{2+} ions reduced the inhibition zones significantly at 5 mmol, but not at 0.5 and 0.05 mmol (Fig.5, p.44).

Al^{3+} ions caused some reduction in the OTC inhibition zones at all three concentrations (Fig. 6, p. 45).

A likely explanation for the reduction of OTC inhibition zones by Al^{3+} and Mg^{2+} ions is the formation of inactive metal chelates with OTC. The higher activity of the Al^{3+} ions may be related to the higher stability constant of 7.0 for the Al^{3+}-OTC complex, as compared

to 3.8 for the Mg^{2+}-OTC complex (Albert and Rees, 1956).

EDTA alone - When incorporated in the assay medium, concentrations of EDTA above 2 mmol inhibited the growth of the test organism. When added to the wells, the EDTA concentration had to exceed 8 mmol before a measurable inhibition zone was observed. Determination by the turbidimetric assay method indicated that EDTA was bacteriostatic between 2 and 5 mmol and bactericidal above 5 mmol.

EDTA and OTC - Addition of EDTA to the assay medium at concentrations between 0.12 and 1.0 mmol increased OTC inhibition zones (Fig. 7, p. 46).

The enhancement of OTC inhibition zones by EDTA is likely to depend on chelation of essential ions for the bacterial growth, thereby causing partial inhibition of the test organism and a synergistic action with OTC (Brown and Richard, 1965; Monkhouse and Grove, 1967; Leive, 1968; Weiser *et al.*, 1969).

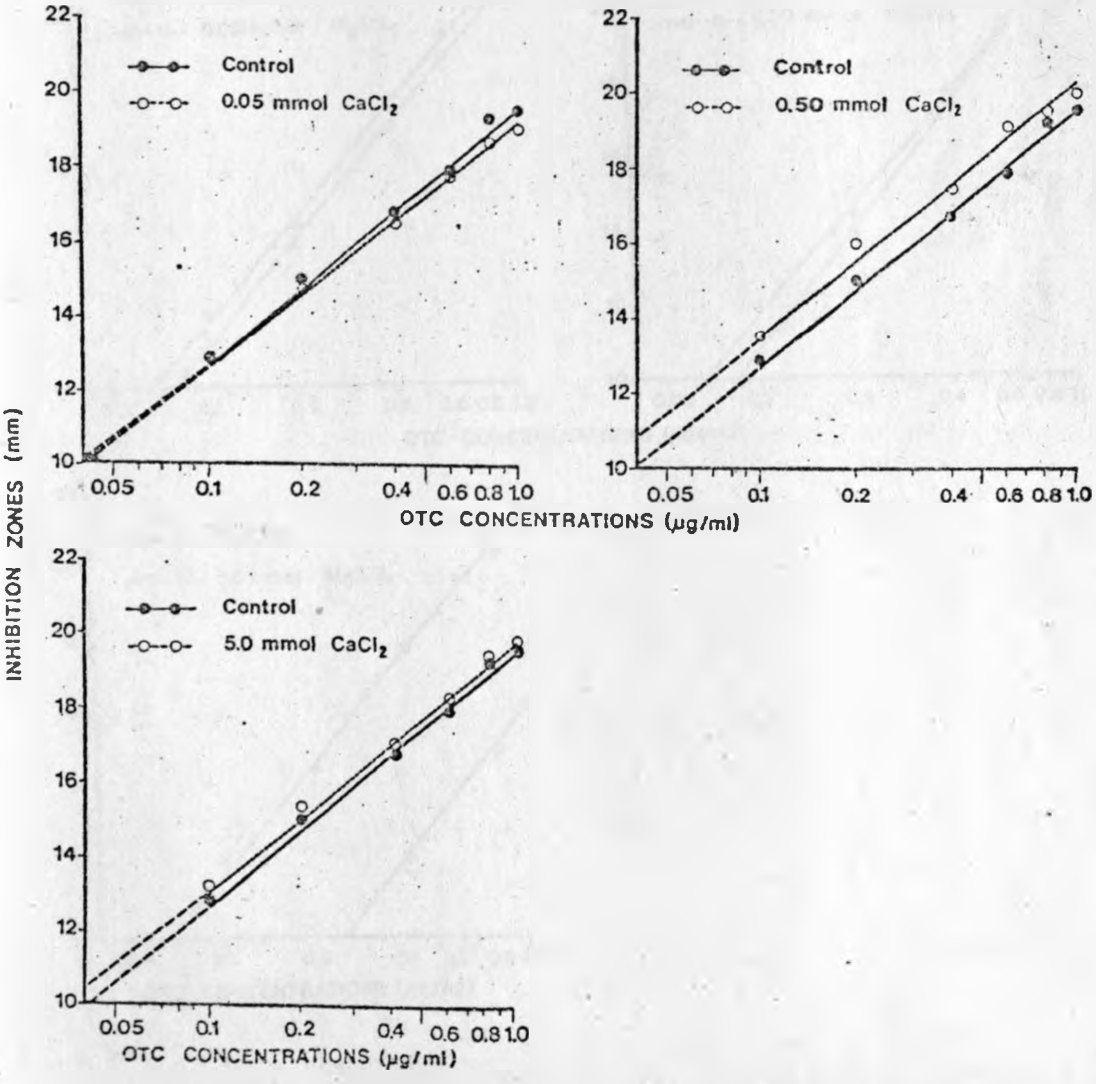


Fig. 4 Effects of various concentrations of calcium chloride on OTC inhibition zones.

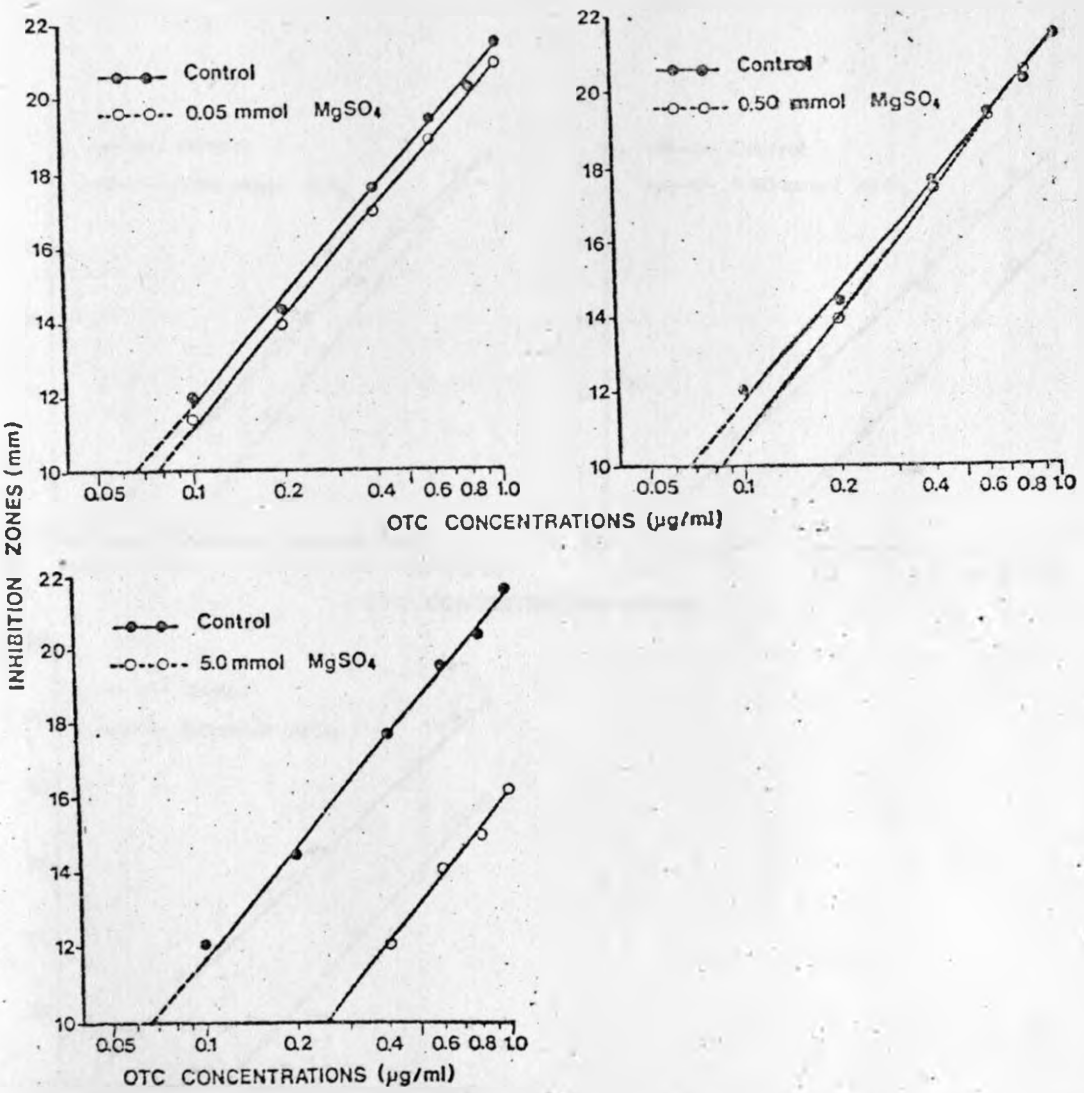


Fig. 5 Effects of various concentrations of magnesium sulphate on OTC inhibition zones.

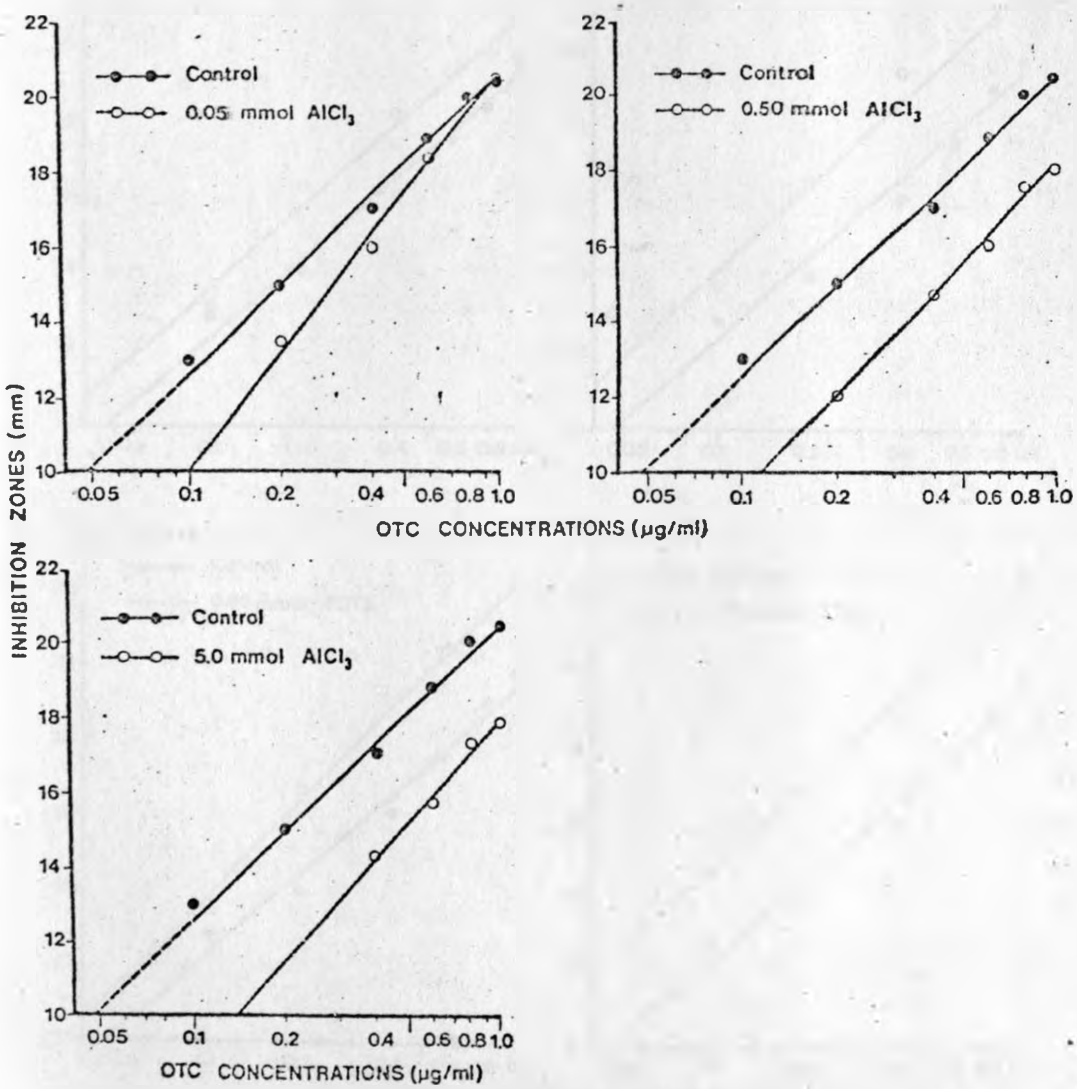


Fig. 6 Effects of various concentrations of aluminium chloride on OTC inhibition zones.

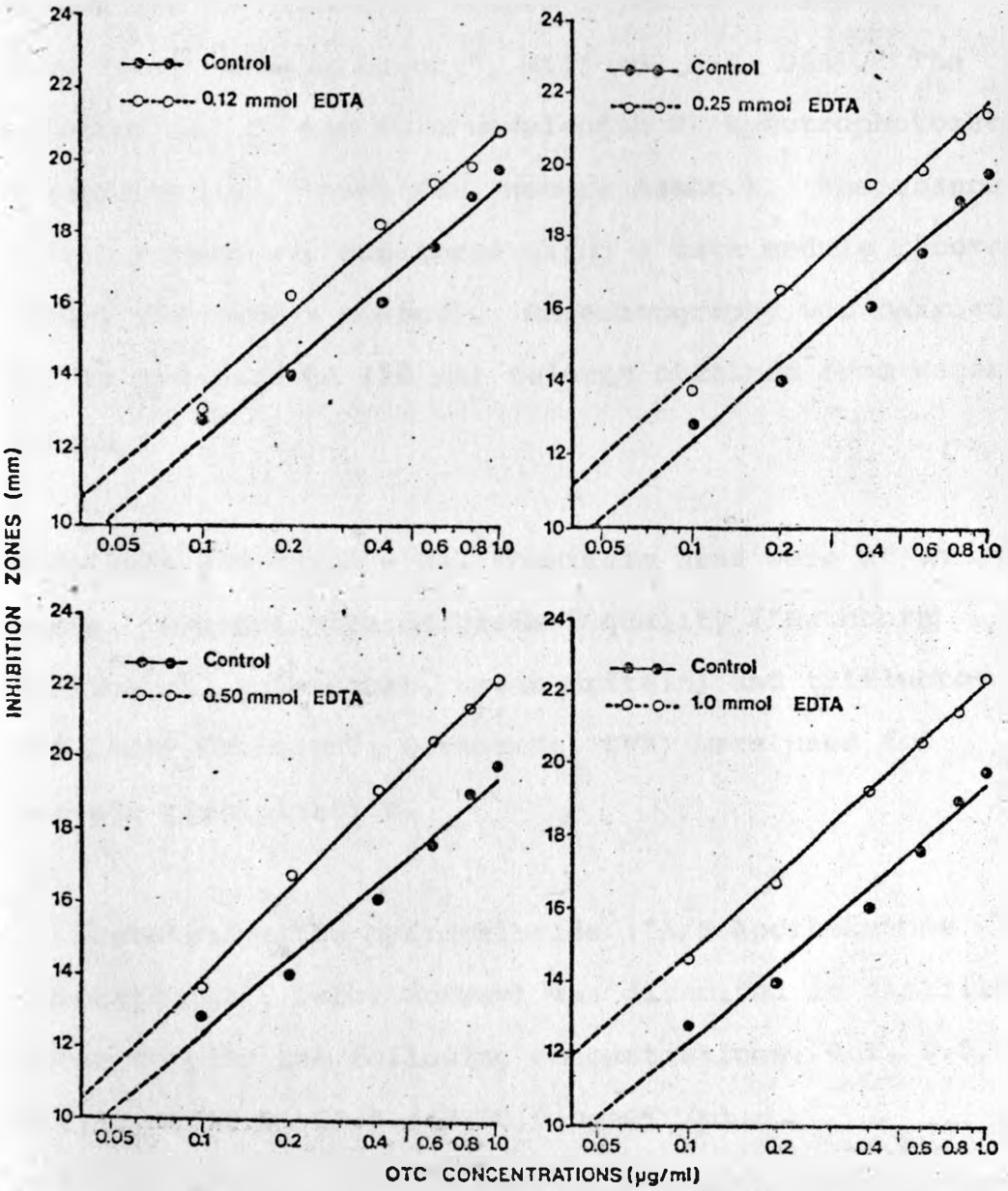


Fig. 7 Effects of various concentrations of EDTA on OTC inhibition zones.

3.5 HIGH PRESSURE LIQUID CHROMATOGRAPHY

3.5.1 Materials and Methods

Equipment - The HPLC consisted of a solvent delivery system and an automatic sample injector (Model 480, Wisp 710B, "Waters Assoc.", Milford, M.A. USA). The detector was a variable wavelength UV-spectrophotometer (Lambda Max Lc, Model 480, Waters Assoc.). Absorbance of the eluent was monitored using a data module recorder (Model 480 Waters Assoc.). Chromatography was carried out in Rad-pack CN (10 µm) columns obtained from Waters Assoc.

Chemicals and drugs - All chemicals used were of analytical grade. Acetonitrile of grade 5 quality ("Rathburn Chemicals", Walkerburn, Great Britain) and trifluoro-acetic acid ("E. Merck", Darmstadt, GFR) were used for protein precipitation.

Oxytetracycline hydrochloride ("A/S Apothekernes Laboratorium", Oslo, Norway) was dissolved in distilled water to give the following concentrations: 0.1, 0.5, 1.0, 5.0, 10.0, 25.0 and 50.0 µg OTC/ml.

Analyses - A standard curve was obtained by injecting 250 µl aliquots of each OTC concentration into the

column. The column was eluted with phosphate buffer, pH 2.4 containing 30% (v/v) acetonitrile at a flow rate of 1.2 ml/min. The pH of the mobile phase was adjusted with orthophosphoric acid (88 - 93%). The eluent was monitored at 357 nm. The chromatograms were recorded automatically at the speed of 0.5 cm/min. The standard curve was prepared by plotting the peak areas against OTC concentrations on arithmetic scale graph paper.

Calculations - The correlation coefficient was calculated from the values of the standard curve according to Yamane (1973).

Recovery studies - OTC was added to antibiotic-free plasma, ruminal fluid and homogenate of dewlap to obtain concentrations between 0.1 and 25 µg OTC/ml. One ml aliquots were then taken and 140 µl trifluoro-acetic acid added for protein precipitation. Thereafter the tubes with the aliquots were agitated for 30 seconds in a whirl mixer before centrifugation for 5 minutes at 5400 g. From the supernatants, 250 µl volumes were taken for injection in the HPLC.

3.5.2 *Results and discussion*

The standard curve for OTC dissolved in water was linear

within the range between 0.1 and 50 µg OTC/ml (Fig. 8, p. 50) with a correlation coefficient of 0.99. About 100% recovery was obtained at a concentration of 25 µg OTC/ml plasma or g dewlap. At the lower OTC concentrations of 0.50 - 5.0 µg OTC/ml, the recoveries were reduced to about 60 - 70%, and at 0.1 µg OTC/ml, only traces could be detected. This method was found to be unsuitable for the analysis of ruminal fluid, since this contained substances which interferred with the spectrophotometric measurement at 357 nm. Modifications aimed at removing these substances were not attempted.

Compared to the time-consuming tetracycline extraction in conventional chemical methods, the HPLC method offers the advantage of only one rapid isolation step (Hermansson, 1982).

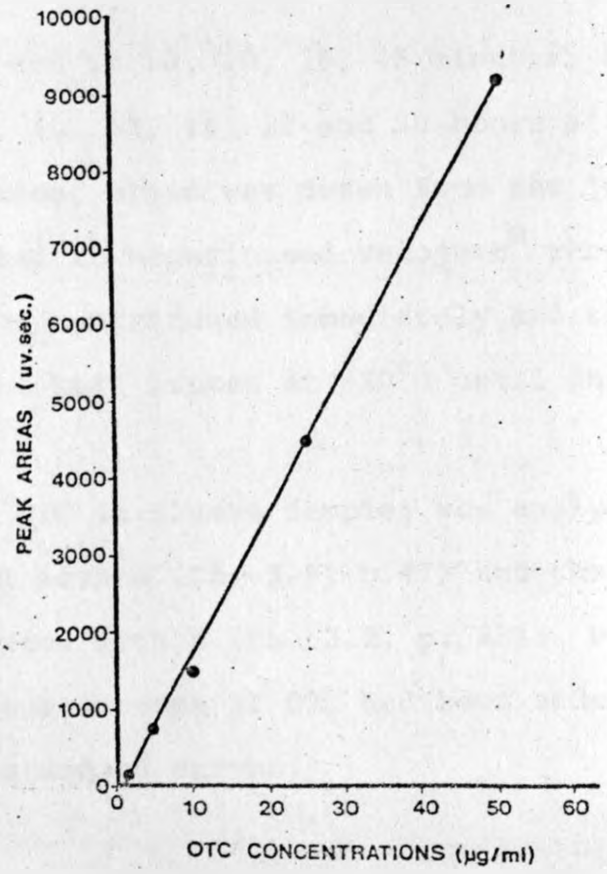


Fig. 8 Standard curve for OTC dissolved in water, obtained by the HPLC method.

3.6 A COMPARISON OF THE HPLC METHOD AND THE MICROBIOLOGICAL ASSAY FOR THE QUANTITATION OF OXYTETRACYCLINE IN PLASMA

3.6.1 Materials and Methods

One adult cow weighing about 500 kg was fitted with a permanent ruminal fistula. The animal was kept indoors and fed on hay and concentrates. The animal was given 10 mg/kg bwt of oxytetracycline (Aquacycline^{R}-5 containing 50 mg OTC/ml, "Rosco", Denmark) by IV injection.

Before, and at 10, 20, 30, 45 minutes; and 1, 1.5, 2, 3, 6, 8, 10, 12, 16, 22 and 30 hours after drug administration, blood was drawn from the jugular vein and collected in heparinised Venoject^{R} tubes. The samples were centrifuged immediately and the plasma obtained was kept frozen at -20^{o}C until analysed.

Analyses - OTC in plasma samples was analysed in parallel by the HPLC method (Ch. 3.5, p.47) and the microbiological agar diffusion method (Ch. 3.2, p. 28). Plasma to which various amounts of OTC had been added was used to obtain standard curves.

3.6.2 Results and discussion

At concentrations between 1.0 and 40.0 µg OTC/ml plasma, there was a good correlation between the two methods

(Fig. 9, p. 53). The correlation coefficient was 0.99.

Nilsson-Ehle *et al.*, (1976) determined tetracycline in sera of humans and dogs using the HPLC and microbiological assay methods and they also found a good correlation.

At lower OTC plasma concentrations, the HPLC method was previously (Ch. 3.5, p. 47) found to give an incomplete recovery of about 60 to 70% between 0.5 to 5.0 µg OTC/ml plasma. Since the two methods gave very similar results, this indicates that the recoveries at lower concentrations were also incomplete with the microbiological agar diffusion method.

OTC half-life, estimated by the HPLC method, was about 11 hours and by the microbiological assay about 10 hours. These values agree well with the OTC half-life of 9.1 hours in cattle reported by Pilloud (1973).

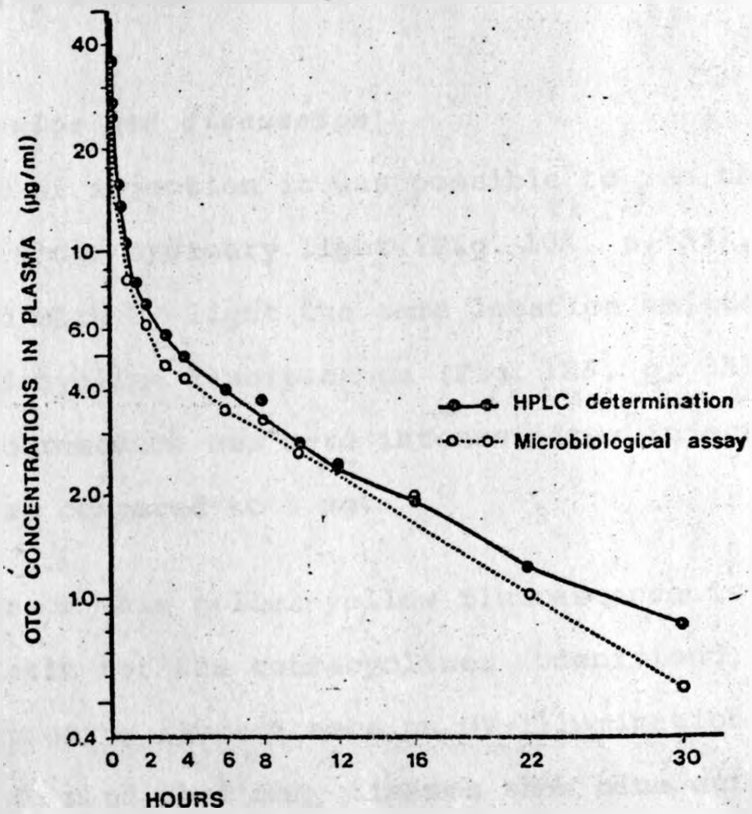


Fig. 9 OTC concentrations (µg/ml) in plasma of a cow following IV injection of Aquacycline^{R}-5 (10 mg/kg bwt) as determined by the HPLC and the microbiological assay methods.

3.7 FLUORESCENCE TECHNIQUE

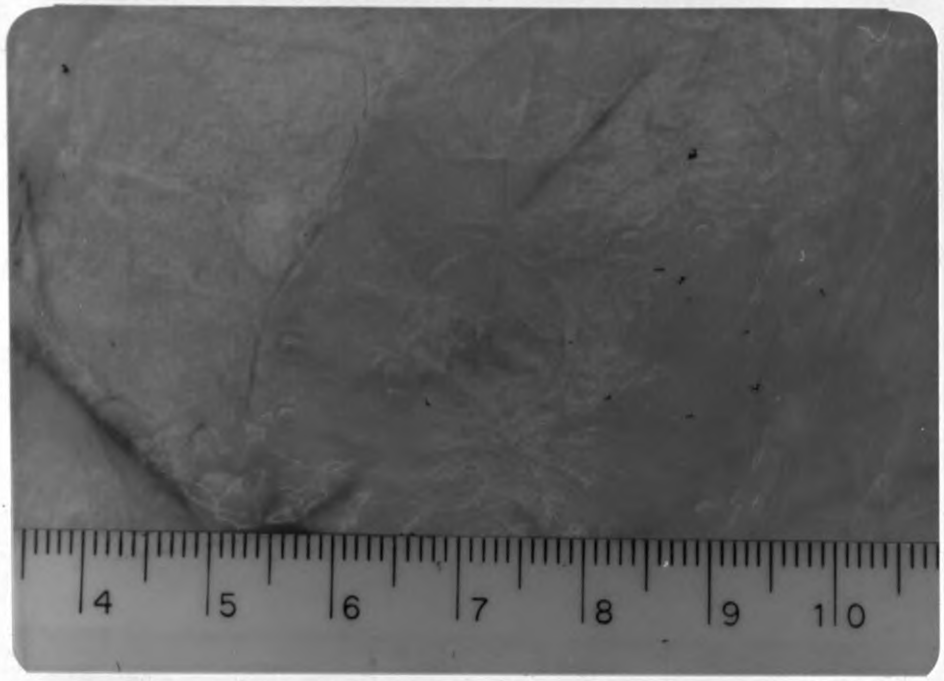
3.7.1 Materials and Methods

Dewlap collected from adult cows after slaughter were injected with 5 and 10 mg of oxytetracycline (Terramycin^{R}-100 injectable solution, 100 mg OTC/ml, "Pfizer", USA). After a diffusion time of 1 hour, the tissues were cut partially through the midline for gross examination and viewing under long wavelength UV-light (Chromato-Vue^{R} "Ultaviolet Products, Inc.", California, USA). Colour photographs were taken with Kodacolor VR film, ASA 200 using a Leica camera.

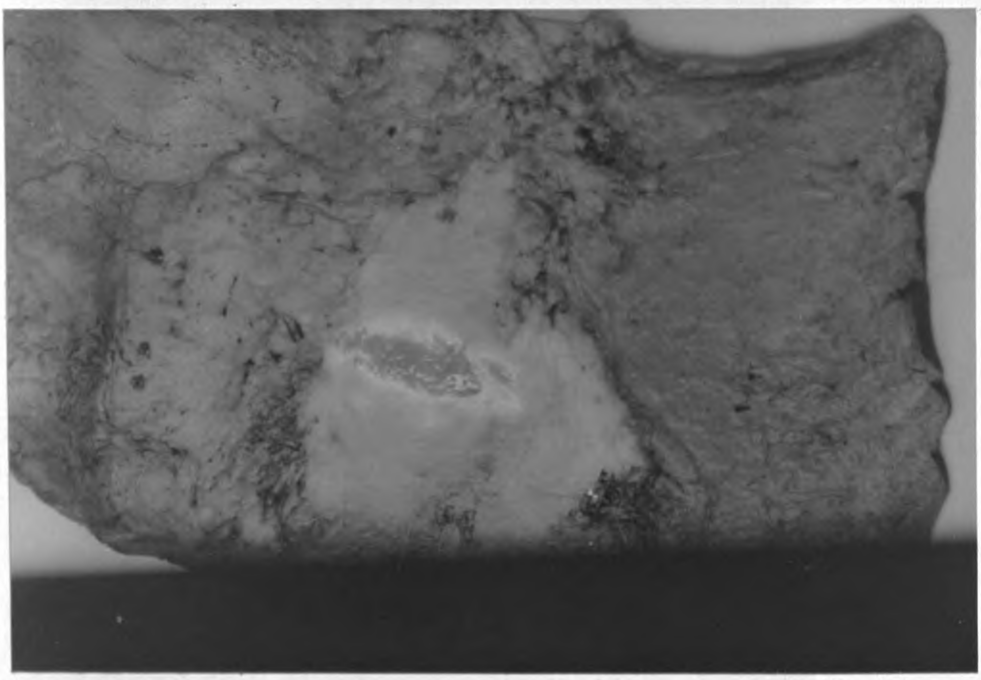
3.7.2 Results and discussion

At the site of injection it was possible to see the yellow OTC colour under ordinary light (Fig. 10*A*, p. 55). When illuminated with UV-light the same location emitted bright yellow tetracycline fluorescence (Fig. 10*B*, p. 55). The OTC fluorescence was more intense after injection of 10 mg OTC as compared to 5 mg.

Emission of this golden-yellow fluorescence is characteristic for the tetracyclines (Udenfriend, 1962). When interpreting fluorescence on UV-illumination, one must bear in mind that many tissues show blue auto-fluorescence, and the liver and bile show a yellow fluorescence similar to that of the tetracyclines (Blomquist and Hanngren, 1966).



A



B

Fig. 10 Photographs of dewlap tissue taken under ordinary light *(A)* and UV-illumination *(B)* after injection of 5 mg oxytetracycline.

3.8 WHOLE-BODY AUTORADIOGRAPHY

3.8.1. Materials and Methods

Fish - Eight rainbow trouts (*Salmo gairdneri*) weighing 200-220 g were used. They were kept in 400-litre fibre glass tanks supplied with fresh running water (1 l/min) at 6±1°C. The fish were fed on commercial fish pellets containing 17% fat ("Landbruskskjemi A/S", Oslo, Norway) during the experiment. They were allowed to adopt to the test conditions for 14 days before the experiment started.

^{3}H-tetracycline (^{3}H-TC) - Tritium-labelled tetracycline (Net - 141 tetracycline {7 - ^{3}H (N)}) was obtained from "New England Nuclear", Boston MA, USA. The specific activity on the date of delivery was 635.4 mCi/mmol. Radiochemical purity was >97% and decomposition on storage (3 months) was <1% at -10°C. ^{3}H - TC was dissolved in 96% ethanol and mixed thoroughly with powdered feed. After evaporation of the alcohol at room temperature, the feed was divided into gelatine capsules No. 2 ("Parke, Davis and Co.", England). One capsule containing 0.14 mg ^{3}H-TC (0.2 mCi) was placed in the stomach of each of six fish by a pair of anatomical tweezers. The fish were watched carefully for 15 minutes to ensure that the capsules were not regurgitated.

Two fish from control group were given unlabelled tetracycline orally.

Autoradiographic procedures - Two, 7 and 21 days after ^{3}H-TC administration, two fish were sacrificed. The two fish serving as controls were sacrificed after 7 days. They were anesthetized in a saturated solution of benzocaine in water and embedded in a gel of 1% carboxymethyl cellulose before being frozen in a bath of hexane and solid carbon dioxide at about -70°C. Sagittal whole-body sections (40µm) were cut at different levels of the body in a PMV cryomicrotome (PMV 450 MP, "Palmstierna Mekaniska Verkstad", Sweden) and collected on tape (No. 821, "3M Co.", St. Paul, Minn., USA) according to Ullberg's autoradiographic technique (Ullberg, 1954). The sections were dried in the cryostat overnight. Autoradiograms were made by opposition of the sections against X-ray films ("LKB Ultrafilm", ^{3}H, Sweden). Exposure was carried out at -20°C for 3 months. After exposure the films were developed in Kodak D 19.

3.8.2 Results and discussion

The patterns of ^{3}H-activity distribution after 7 and 21 days are shown in Figs. 11, p. 59 and 12, p. 60). The major part of the radioactivity was located in the

gastrointestinal tract, liver, gall bladder, bones and the skin, where substantial amounts persisted after 21 days. Higher concentration, but essentially the same pattern was observed after two days. On no occassion was any radioactivity observed in the brain.

In mammals, affinity of tetracycline for bones and keratinised tissues, as well as enterohepatic recirculation, have been reported by many investigators (e.g. André, 1956). The present experiment which appears to be the first on the pharmacokinetics of tetracyclines in fish, demonstrates a pattern of distribution and accumulation which is essentially the same as in mammals.

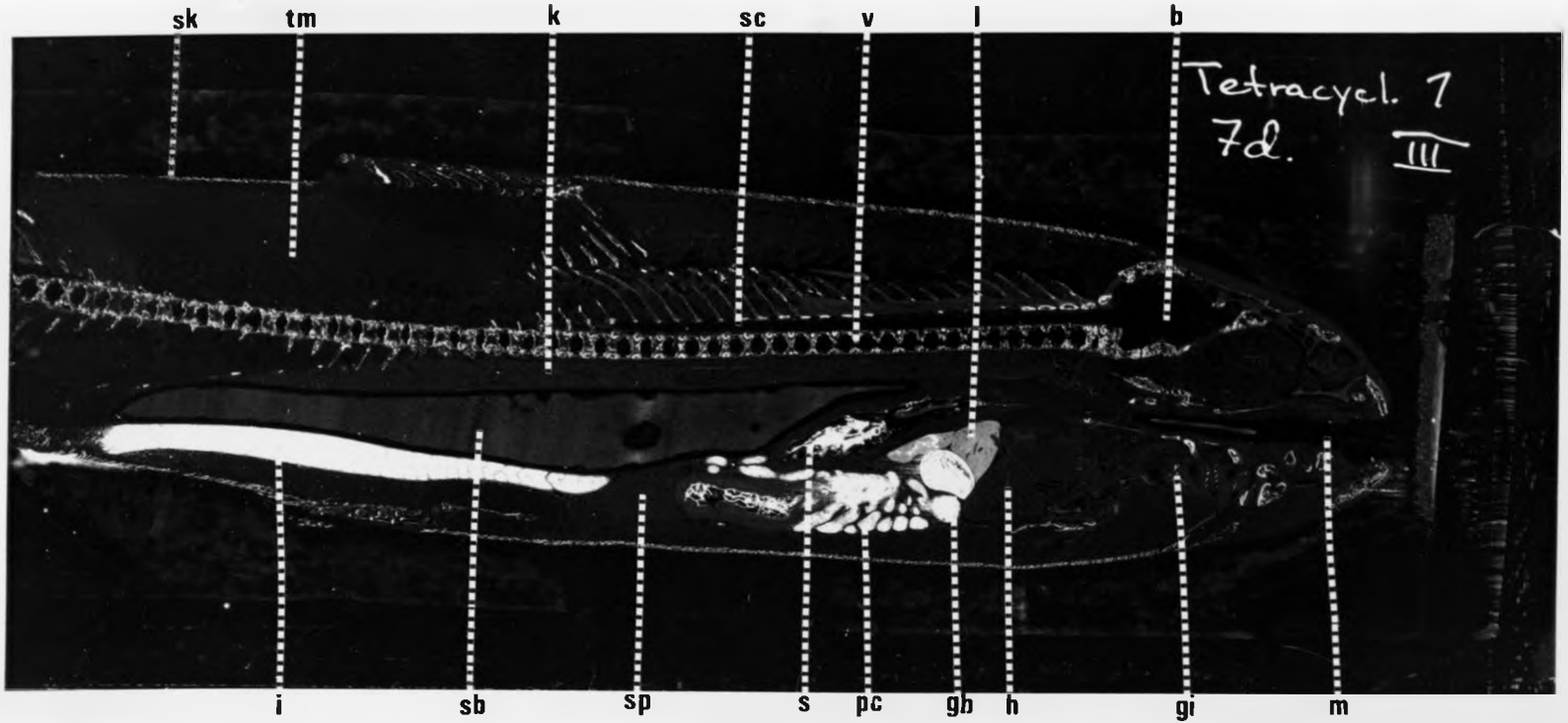


Fig. 11 Distribution of ^{3}H - activity in rainbow trout, 7 days after ^{3}H - tetracycline administration (0.14 mg TC 0.2 mCi).

sk = skin, *tm* = trunk muscle, *k* = kidney, *sc* = spinal column, *v* = vertebra, *l* = liver
b = brain, *m* = mouth, *gi* = gills, *h* = heart, *gb* = gall bladder, *pc* = pyloric caeca,
s = stomach *sp* = spleen *sb* = swim bladder *i* = intestine

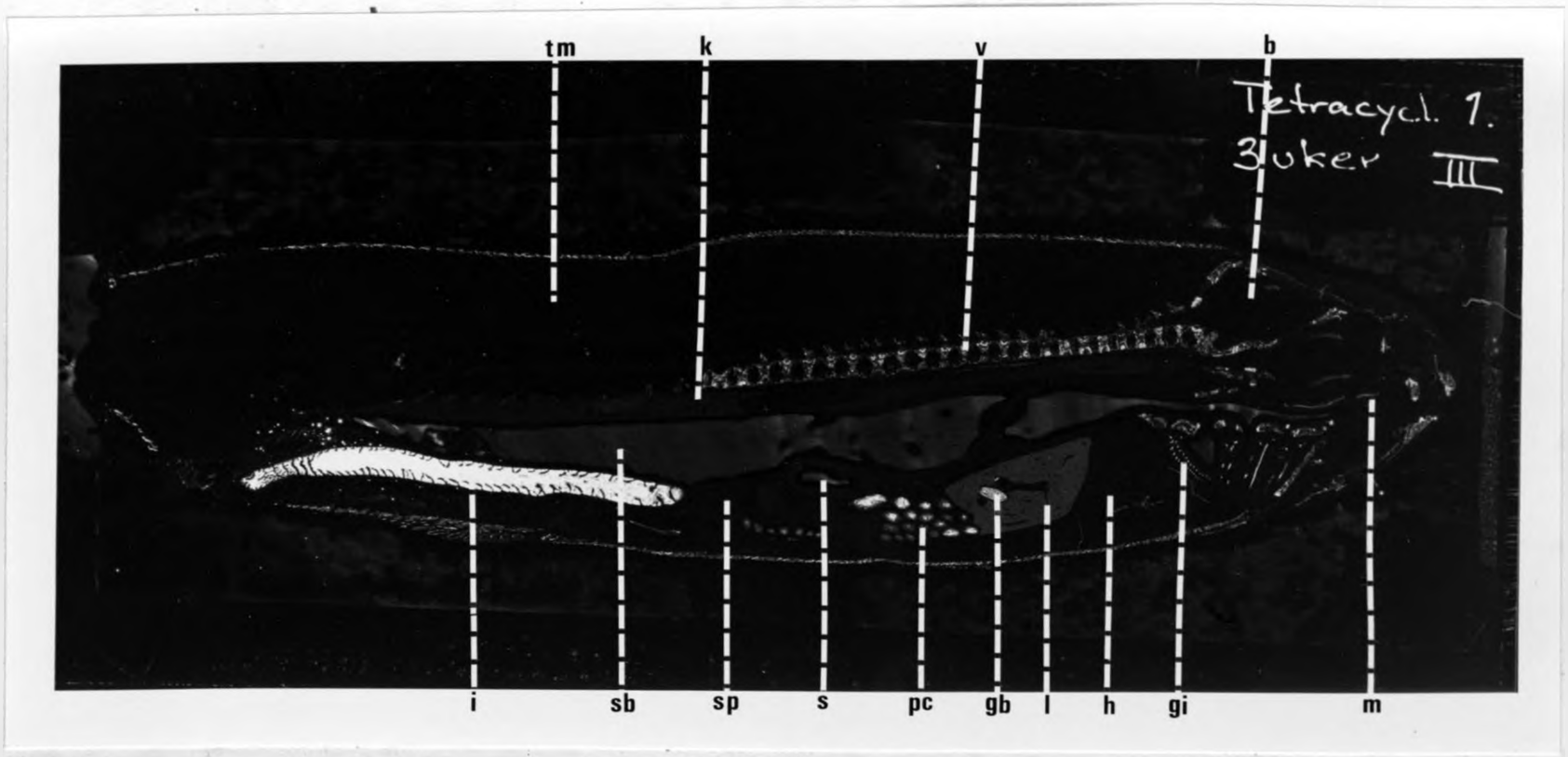


Fig. 12 Distribution of ^{3}H - activity in rainbow trout, 21 days after ^{3}H - tetracycline administration (0.14 mg TC, 0.2 mCi).

tm = trunk muscle, *gb* = gall bladder, *pc* = pyloric caeca, *sb* = swim bladder *k* = kidney
v = vertebra *b* = brain *m* = mouth *gi* = gills *h* = heart
l = liver *s* = stomach *sp* = spleen *i* = intestine

CHAPTER FOUR

A COMPARISON OF A CONVENTIONAL (TERRAMYCIN^R-100) AND A LONG-ACTING (TERRAMYCIN^R/LA) OXYTETRACYCLINE FORMULATION WITH RESPECT TO SERUM CONCENTRATIONS, LOCAL TISSUE REACTIONS AND RESIDUES AFTER INTRAMUSCULAR AND DEWLAP INJECTIONS IN CALVES

4.1 INTRODUCTION

Injectable oxytetracycline (OTC) products for animal use are available either in powder form to be reconstituted with water immediately before treatment or in a ready-to-use form in which the drug is solubilized in organic solvents. Most users prefer the latter forms because of convenience, longer shelf life and economic considerations (Cornwell, 1980; Ziv, 1980).

With conventional OTC formulations, such as Terramycin^R - 100 injectable solution ("Pfizer", USA), repeated daily injections over a period of 3 to 5 days are necessary to maintain adequate serum levels. Assuming a therapeutic minimum serum concentration of 0.5 µg OTC/ml, this will be maintained for about 24 hours following intramuscular (IM) injection of the highest recommended dose of 10 mg OTC/kg bwt. (Luthman and Jacobsson, 1982).

A long-acting OTC preparation, now marketed for

veterinary use, Terramycin^{R}/LA long-acting injectable solution ("Pfizer", USA) is claimed to reduce the need for frequent injections and thereby lower the costs. According to the manufacturers, serum concentrations of OTC above 0.5 µg/ml are maintained for 3 to 5 days after a single IM injection of 20 mg OTC-LA/kg bwt. The prolonged effect of OTC-LA is claimed to be due to the use of an aqueous 2 - pyrrolidone - based formulation which should lead to a slow release of OTC at the injection site without significant tissue damage (Simpson, 1978; Cornwell, 1980). Intramuscular administration of a number of drugs is known to cause local tissue reactions at the site of injection. Severe local reactions with necrosis and haemorrhage were observed in cows six days after IM injection of OTC (Rasmussen and Høgh, 1971). Depot formulations are prone to give higher and more persistant levels of residues than conventional preparations. This has considerable implications when stipulating pre-slaughter time limits after drug administration. Furthermore, the local tissue reactions in muscles may be sufficiently severe to result in the condemnation of a considerable portion of muscle during meat inspection.

An alternative injection site thus warranted consideration. The dewlap is a skin fold of no economic value with abundant loose subcutaneous connective tissue which can easily be removed during meat inspection.

The present trial was aimed at investigating the suitability of deep dewlap injection as an alternative to IM injection of OTC, by comparing a conventional and a long-acting preparation with regard to serum concentrations, tissue reactions and drug residues at the sites of injection.

4.2 MATERIALS AND METHODS

4.2.1 Animals

The trial included eight mixed breed female calves with a mean age of 8 months (range 6 - 13 months) and an average weight of 142 kg (range 109 - 191 kg) at the beginning of the experiment.

4.2.2 Drugs

The two oxytetracycline (OTC) preparations were:

OTC-LA: Terramycin^{R}/LA injectable solution containing 200 mg OTC/ml ("Pfizer International, Inc.", New York, USA)

OTC-C : Terramycin^{R} - 100 injectable solution containing 100 mg OTC/ml ("Pfizer International, Inc.", New York, USA).

According to the randomisation scheme shown in Table 7, p. 64, each animal received two injections of 20 mg OTC/kg bwt of either OTC-LA or OTC-C, one IM and the other in the dewlap. The interval between the two injections was two weeks. The IM injections were given in the neck region, a hand width cranially to the scapula. The dewlap injections were introduced deeply towards the centre of the

dewlap.

4.2.3 Blood samples

Ten ml blood samples were collected from the jugular vein using non-heparinised vacutainers (Venoject^{R}, "Terumo Corporation", Japan). Samples were drawn just before, and at 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120 and 144 hours after drug administration. The blood samples were allowed to clot and centrifuged to obtain serum samples which were kept at -20^{o}C until assayed for OTC.

Table 7 Randomisation of IM and dewlap injections in a cross-over trial with 8 calves. With an interval of two weeks, each calf received the two injections of 20 mg OTC/kg bwt as either OTC-C or OTC-LA

Calf No.	Body weight, kg	Injection volume, ml	Site of first injection	Site of second injection	Preparation
1	145	29.0	dewlap	IM	OTC-C
5	191	19.1	dewlap	IM	OTC-LA
2	143	28.6	IM	dewlap	OTC-C
6	143	14.3	IM	dewlap	OTC-LA
3	109	21.8	dewlap	IM	OTC-C
7	143	14.3	dewlap	IM	OTC-LA
4	129	25.8	IM	dewlap	OTC-C
8	135	13.5	IM	dewlap	OTC-LA

4.2.4 Registration of swelling

The degree of swelling in the dewlap was estimated by simple subtraction of measurements of the dewlap thickness at the site of injection, made before and at the stated intervals after injection. A vernier gauge was used for the measurement. The reactions at the IM injection sites were examined by palpation.

4.2.5 Tissue samples

Two calves (Nos. 4 and 8) were slaughtered 30 days, and two other calves (Nos. 2 and 6) 49 days after injection. Large portions of the neck muscle and the dewlap were excised around the sites of injection.

The dewlap injection sites were exposed by cutting partially through the midline for macroscopic examination and photography (Ch. 3.7, p. 54). The neck injection sites were examined in the same way after incisions had been made along the muscle fibres to examine for possible morphological changes. The sites of injection were further illuminated with UV-light for the detection of OTC and photography. The areas showing tetracycline fluorescence and tissue damage were excised and weighed, and from these, representative sections were cut out for histological examination and for OTC assays. The samples for OTC analyses were kept at -20^{o}C until analysed, and those for histology were preserved in 10% buffered formalin.

4.2.6 OTC analyses

OTC concentrations in serum samples were determined by the microbiological assay method, while OTC in the tissue samples was quantitated by both the microbiological assay and HPLC method (Ch. 3.2, p. 28 and Ch. 3.5, p. 47).

4.2.7 Histology

The specimens for histology were embedded in paraffin wax and sections were cut and stained with haematoxylin and eosin (HE) for microscopic examination.

4.2.8 Calculations

The areas under the OTC serum concentration - time curves (AUCs) were calculated by the trapezoidal method (Baggot, 1977):

$$AUC = \int_0^{\infty} Cpdt = \int_0^{t^*} + \frac{Cp(t^*)}{\beta}$$ where

AUC = area under serum concentration - time curve

Cp = serum concentration of drug at t

t = time after drug administration

t* = time at which last sample was collected for analysis

β = overall elimination rate constant ($0.693/t_{\frac{1}{2}}$) with $t_{\frac{1}{2}}$ being the half-life of the drug

Cp(t*) = last measured serum concentration of the drug

Student's t-test was used for statistical comparison between the mean values of the calculated AUCs (Durant, 1977).

The concentrations of OTC in serum and tissue samples were calculated as described in Chapter 3 (3.3, p. 35). The concentrations of OTC residues in the selected representative samples showing fluorescence and the total weight of this part, served as basis for obtaining a rough estimate of OTC residues at the injection sites.

4.3 RESULTS

4.3.1 OTC serum concentrations

Intramuscular injections of OTC-C and OTC-LA resulted in OTC serum concentration curves with some individual differences (Fig. 13, p. 70), but the mean serum concentration curves were fairly similar (Fig. 14, p. 71). Both reached peak concentrations after about 4 hours. It was slightly higher after OTC-LA, 7.5 ± 2.5 µg OTC/ml, as compared to 6.7 ± 2.2 µg OTC/ml after OTC-C. Concentrations above 0.5 µg OTC/ml were maintained for 52 hours (range 49 - 64 hours) after OTC-LA, compared to 45 hours (range 42 - 51 hours) after OTC-C. The difference between the AUCs proved not to be significant ($p > 0.10$), see Appendix 1, p. 146.

Injections in the dewlap gave also similar mean serum concentration curves for the two preparations (Fig. 14, p. 71), but it took about 12 hours before the peak concentrations were reached. These were lower than after IM injection, being only 1.7 ± 0.3 and 1.8 ± 0.2 µg OTC/ml after OTC-LA and OTC-C respectively. The

concentrations remained above 0.5 µg OTC/ml for 62 hours (range 52 - 69 hours) after OTC-LA and for 57 hours (range 50 - 59 hours) after OTC-C. The difference between the two preparations with regard to AUCs, was also significant after dewlap injection ($p > 0.10$), see Appendix 1, p. 146.

There were large and significant differences ($p < 0.01$), however, between the serum concentrations after dewlap and IM injections for both OTC-LA and OTC-C (Fig. 13, p. 70 and Fig. 14, p. 71). Within the first 24 hours, the mean AUCs after dewlap injection were only 34 and 32% of those after IM injection of OTC-C and OTC-LA respectively.

4.3.2 Clinical observations and swelling

It was evident that the calves experienced more pain during IM injection compared with the injection in the dewlap. Swellings palpated after IM injection revealed no obvious difference between the two OTC preparations. With the exception of calf No. 1, the calves which received dewlap injection of OTC-C showed less swellings compared with the calves given OTC-LA (Fig. 15, p. 72).

4.3.3 Necropsy

Macroscopic examination of IM injection sites 44 and 63 days after injection revealed no pathological changes

neither after OTC-C nor after OTC-LA. At the dewlap injection sites necrosis was observed 30 (Fig. 16, p. 73) and 49 days (Fig. 17, p. 74) after injection, the areas of necrosis were much more pronounced in those calves which received OTC-LA (Figs. 16B and 17B).

Microscopic examination of the dewlap injection sites revealed areas of coagulative necrosis surrounded by inflammatory cells and fibrosis 30 and 49 days after injections of OTC-C and OTC-LA (Fig. 18, p. 75 and Fig. 19, p. 76). The histopathological changes were more pronounced in lesions caused by OTC-LA (Figs. 18B and 19B). At the IM injection sites small areas of irregular patterns of the muscle fibres 44 days after injections of both OTC preparations were observed (Fig. 20, p. 77).

4.3.4 OTC residues at the injection sites

Neither UV-illumination nor the microbiological assay or the HPLC analyses, were able to detect OTC residues at the IM injection sites 44 and 63 days after administration. When the dewlap injection sites were illuminated by UV-light, yellow fluorescence demonstrated the presence of OTC (Fig. 21, p. 78 and Fig. 22, p. 79). The fluorescence was more intense after OTC-LA administration (Figs. 21B and 22B). The total amount of OTC residues were estimated to be 2.1 mg 30 days after and 1.6 mg 49 days after injection of OTC-LA in the dewlap. The corresponding values for OTC-C were 0.3 and 0.1 mg. Identical results were obtained with both the microbiological assay and the HPLC method.

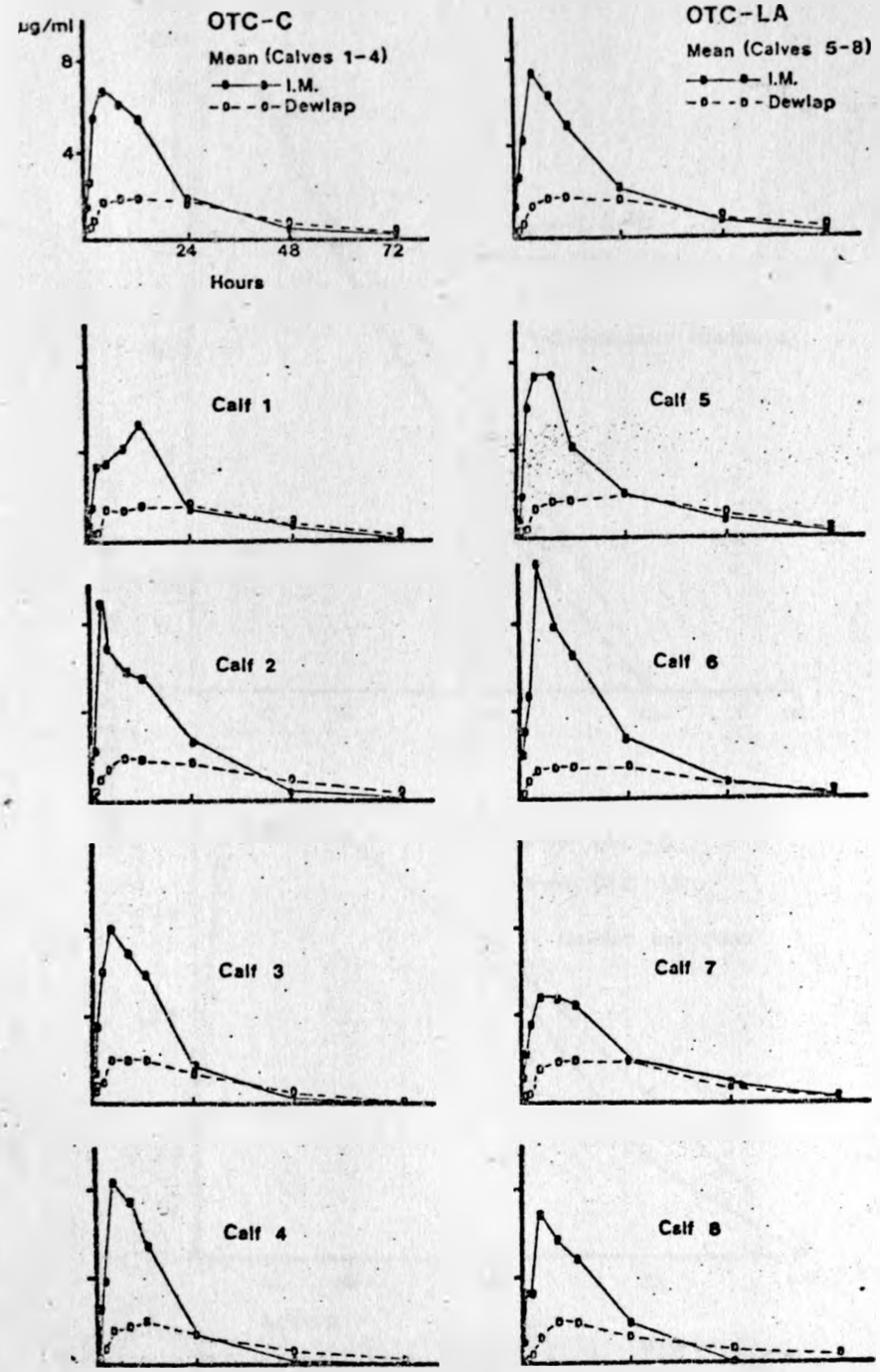


Fig. 13 Mean and individual serum concentrations of OTC (µg/ml) after IM and dewlap injection of OTC-C and OTC-LA, 20 mg/kg bwt in a cross-over trial with 8 calves.

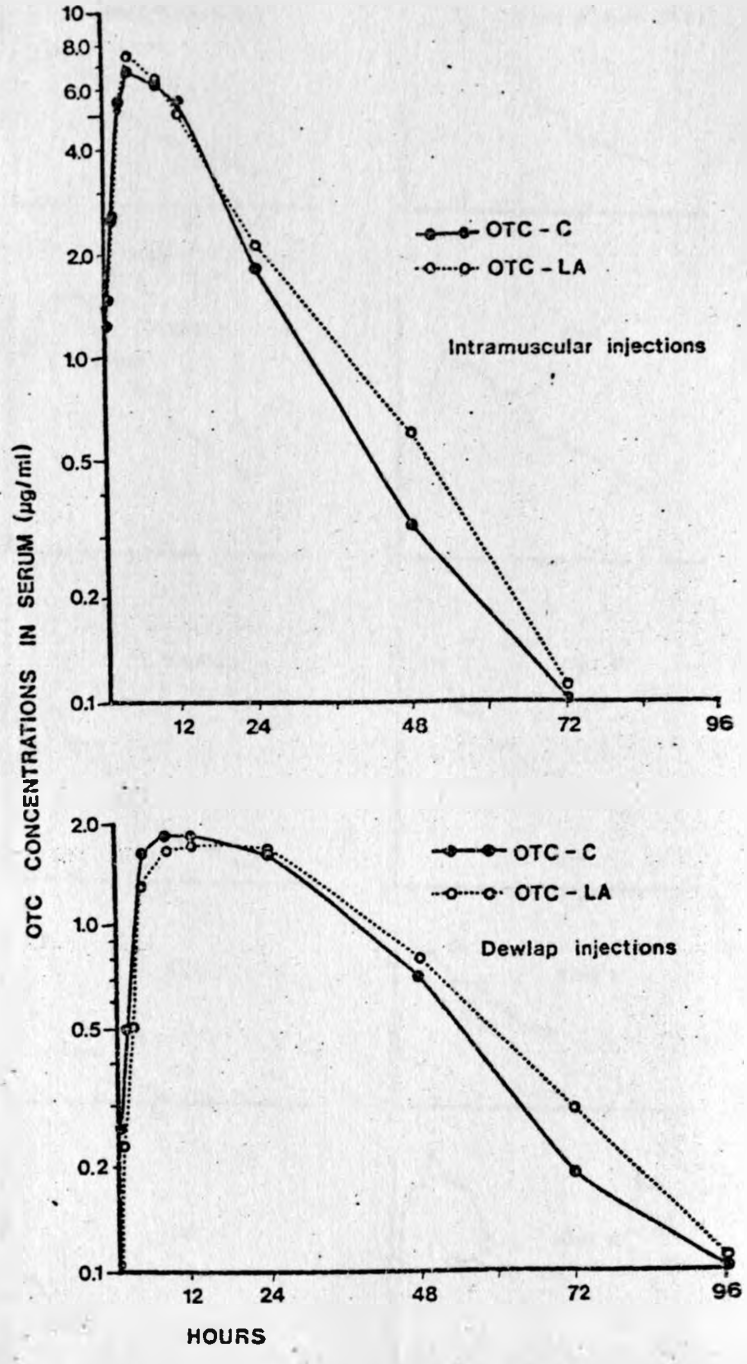


Fig. 14 Mean OTC serum concentrations (µg/ml) after IM and dewlap injections of OTC-C and OTC-LA, 20 mg/kg bwt in a cross-over trial with 8 calves.

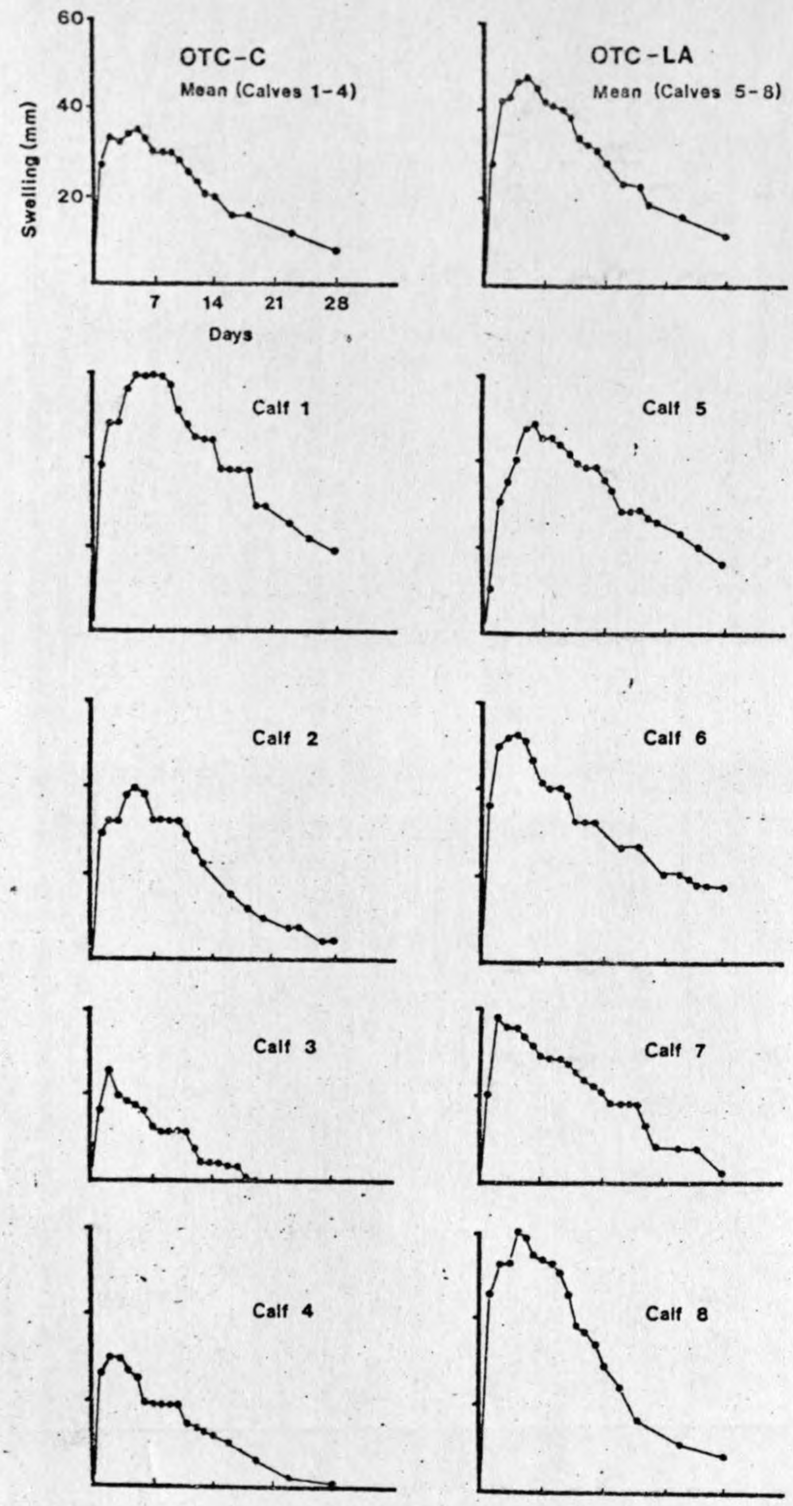


Fig. 15 Mean and individual swellings in the dewlaps (mm) after dewlap injections of OTC-C and OTC-LA, 20 mg/kg bwt, in a cross-over trial with 8 calves.

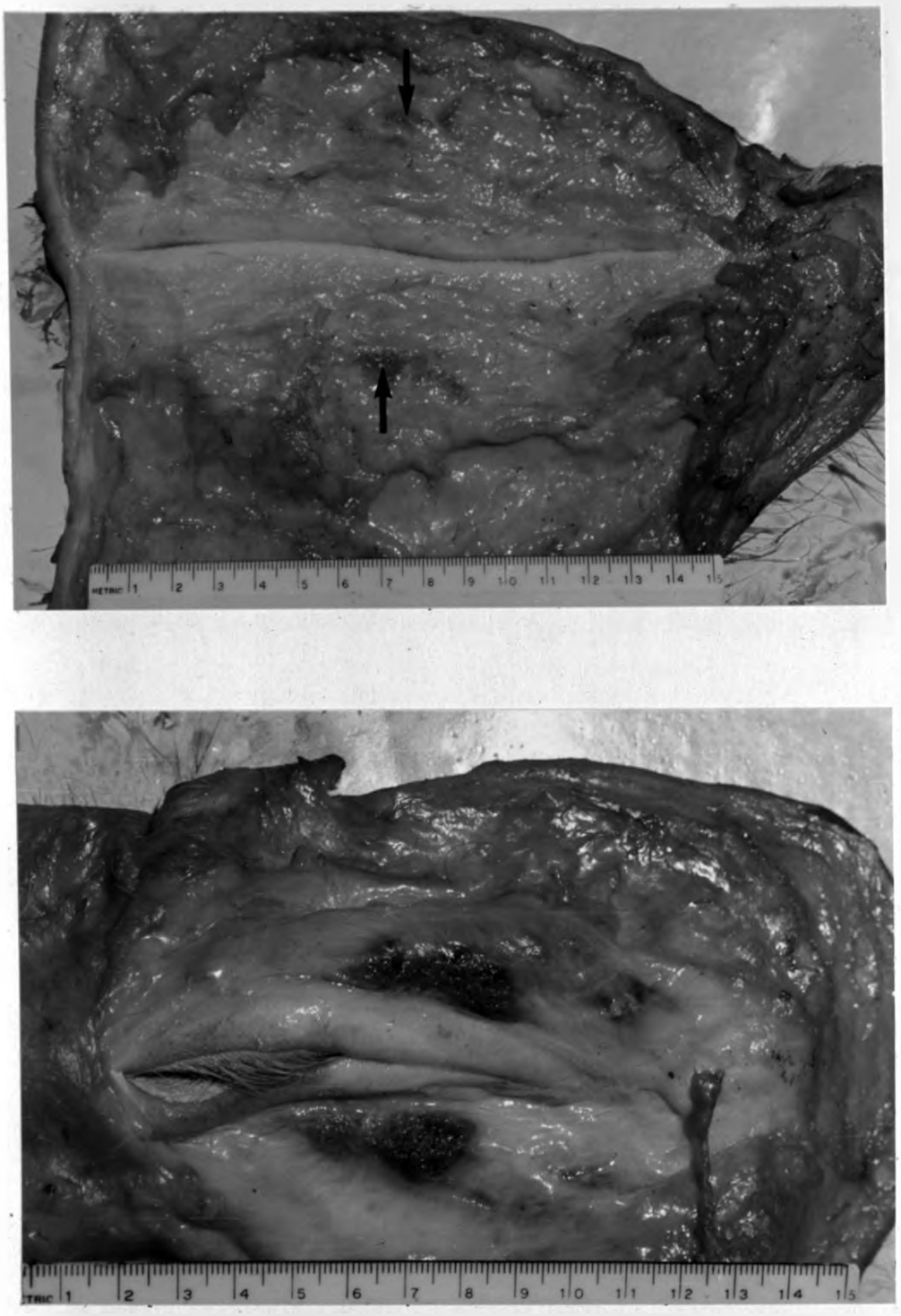


Fig. 16 Photographs of dewlap injection sites taken under ordinary light 30 days after injection of *(A)* 2580 mg OTC-C (25.8 ml) and *(B)* 2700 mg OTC-LA (13.5 ml).

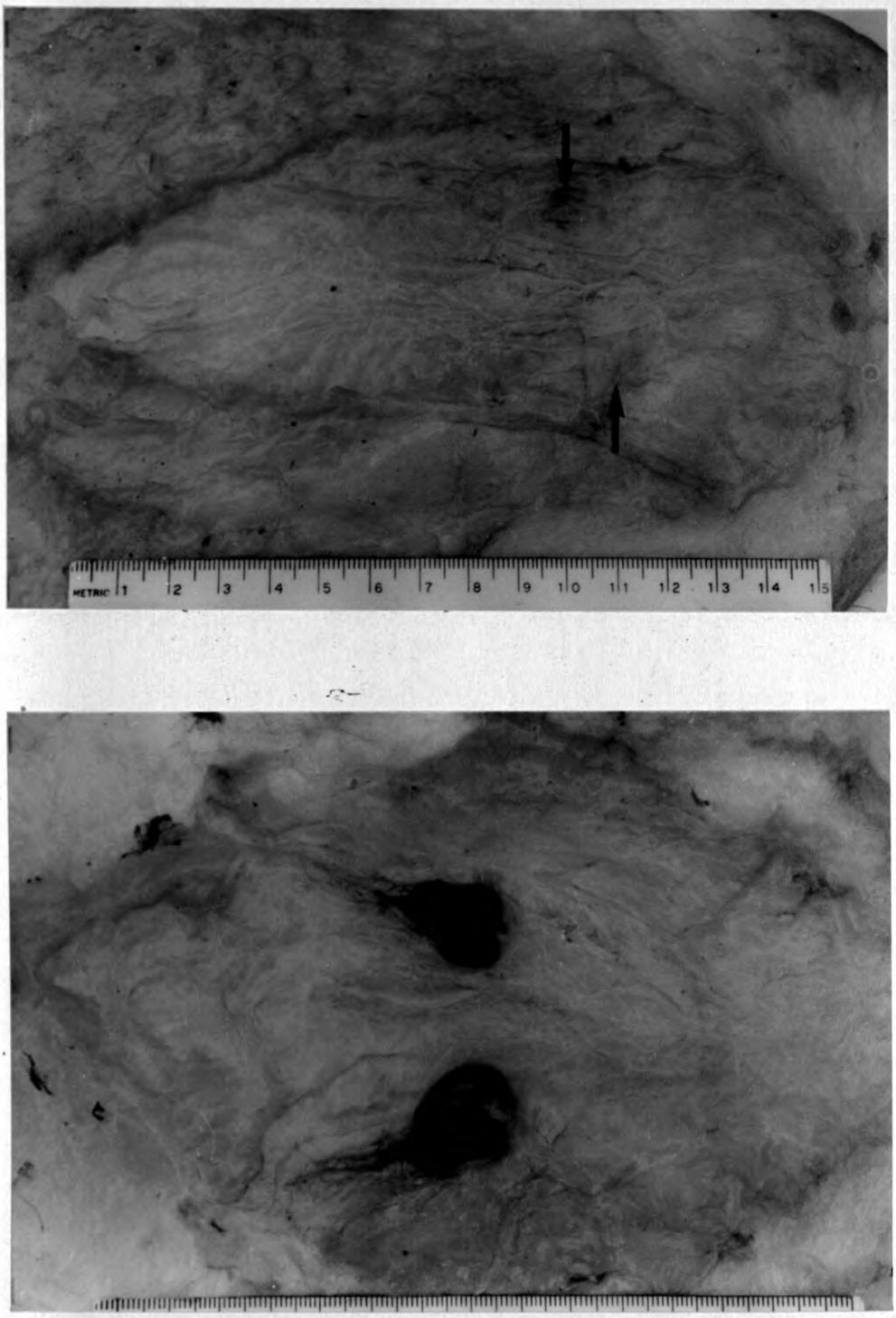


Fig. 17 Photographs of dewlap injection sites taken under ordinary light 49 days after injection of *(A)* 2860 mg OTC-C (28.6 ml) and *(B)* 2860 mg OTC-LA (14.3 ml).

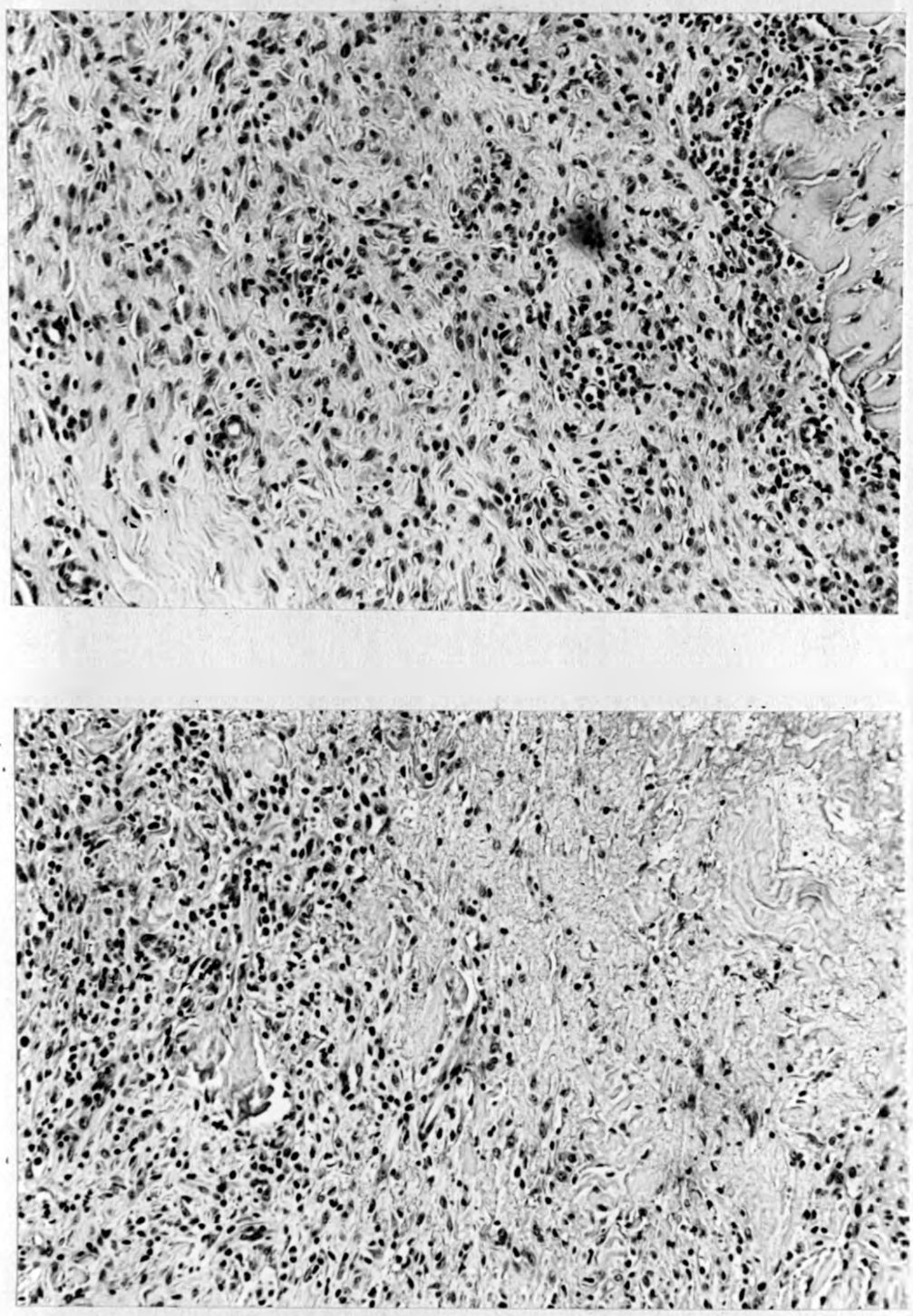


Fig. 18 Photographs of the histological sections of dewlap injection sites 30 days after injection of *(A)* 2580 mg OTC-C (25.8 ml) and *(B)* 2700 mg OTC-LA (13.5 ml) 170 x HE.

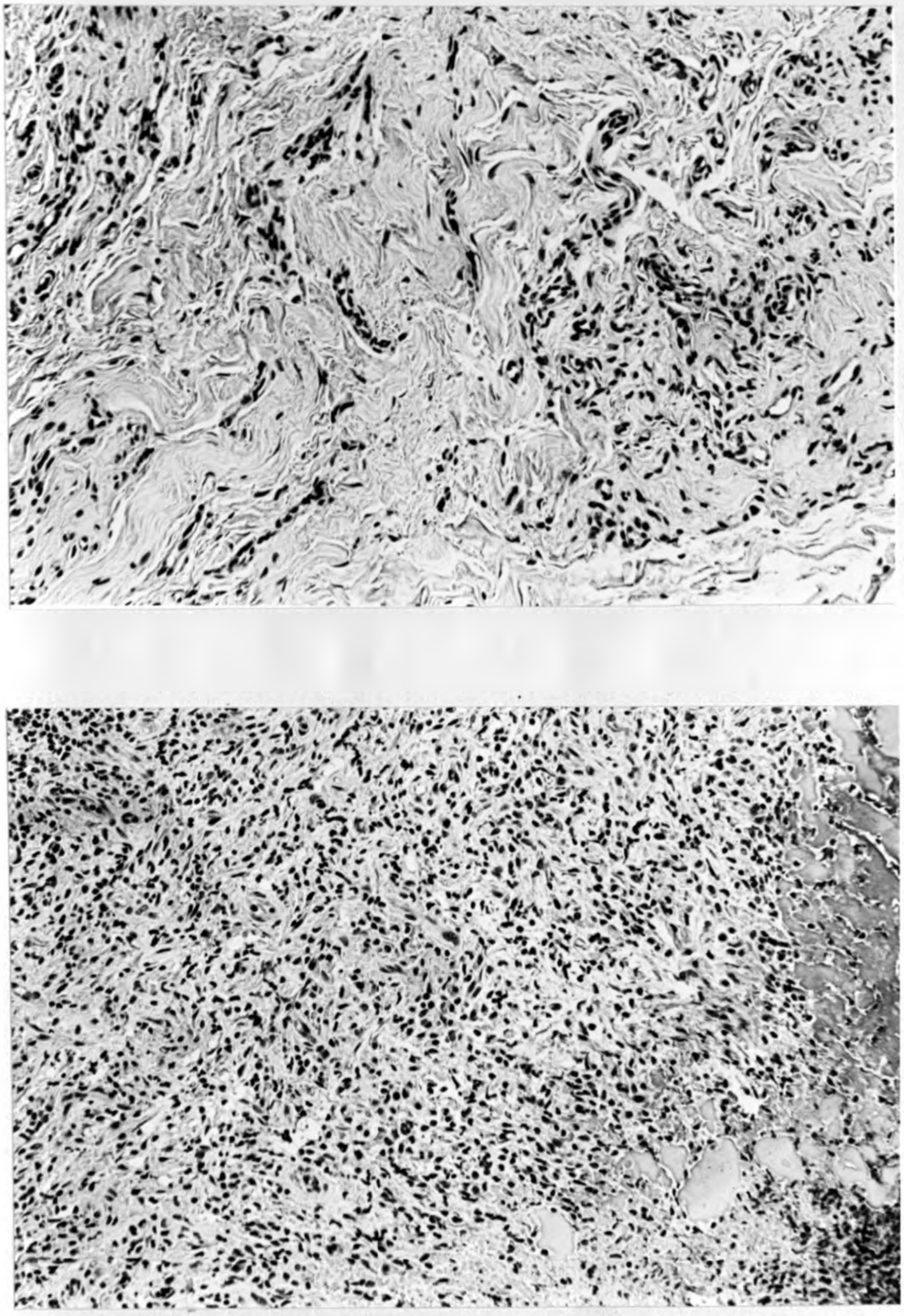


Fig. 19 Photographs of the histological sections of dewlap injection sites 49 days after injection of *(A)* 2860 mg OTC-C (28.6 ml) and *(B)* 2860 mg OTC-LA (14.3 ml) 170 x HE.

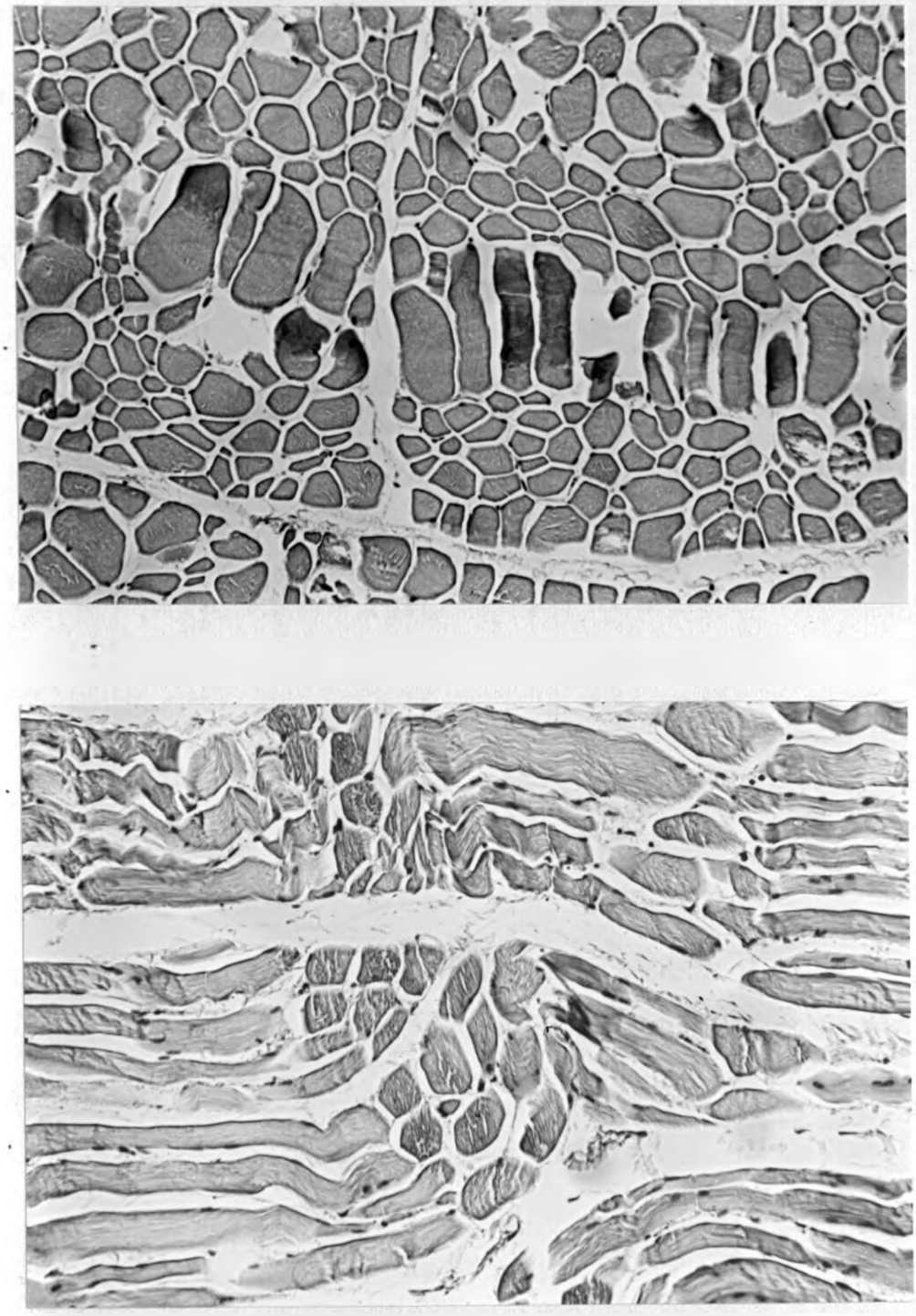


Fig. 20 Photographs of the histological sections of IM injection sites 44 days after injection of *(A)* 2580 mg OTC-C (25.8 ml) and *(B)* 2700 mg OTC-LA (13.5 ml) 170x HE.

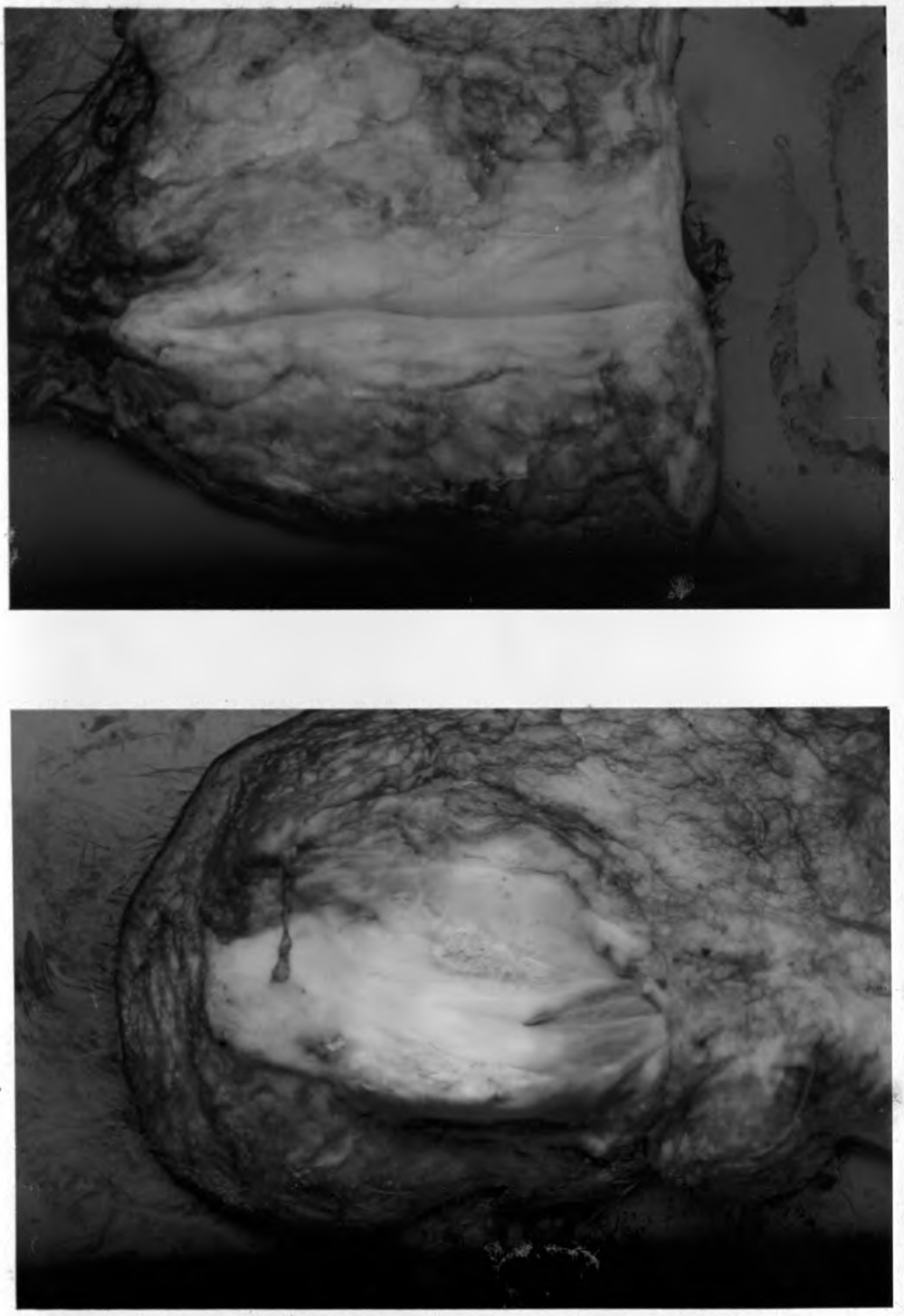


Fig. 21 Photographs of dewlap injection sites taken under UV-illumination 30 days after injection of *(A)* 2580 mg OTC-C and *(B)* 2700 mg OTC-LA.

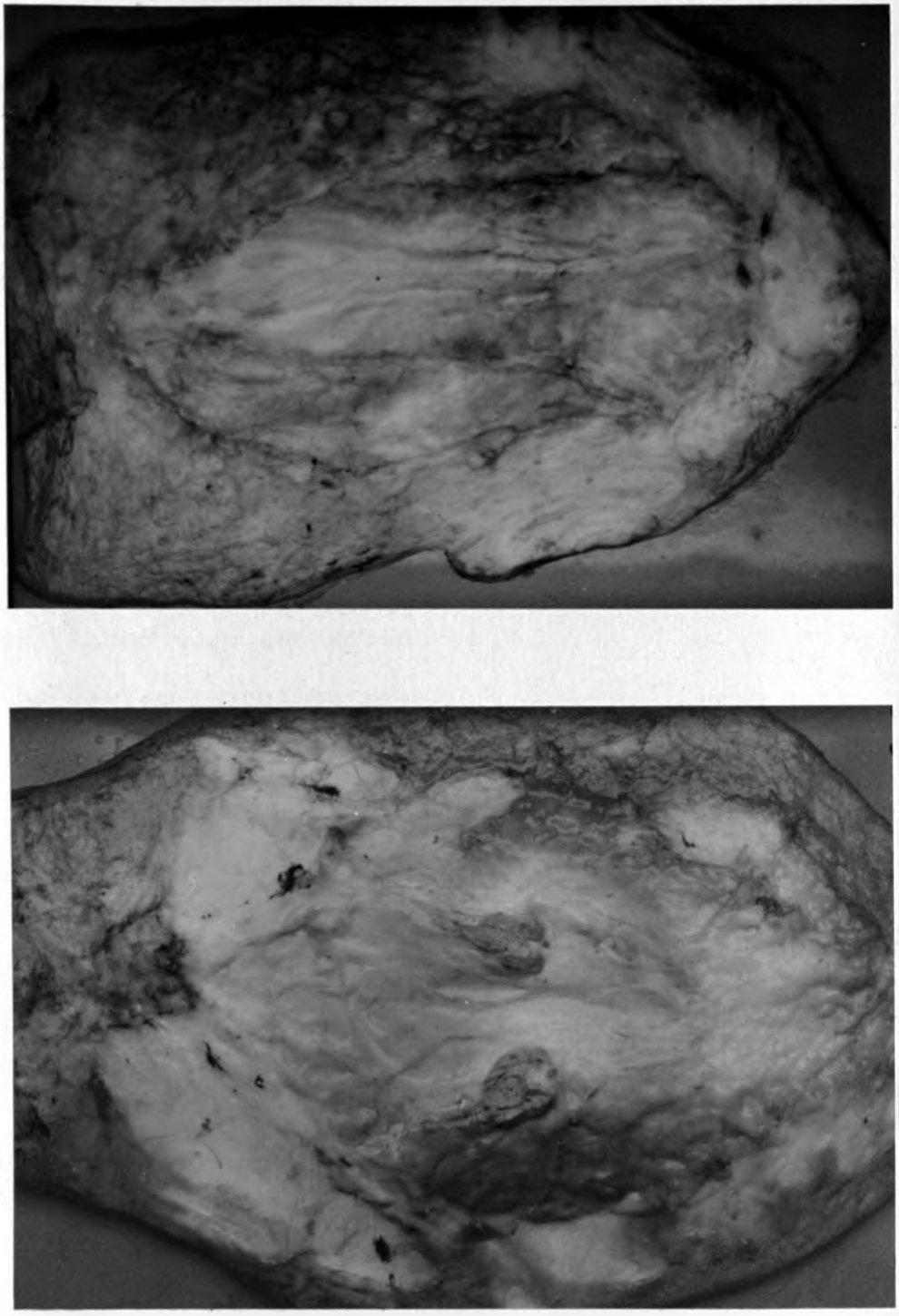


Fig. 22 Photographs of dewlap injection sites taken under UV-light 49 days after injection of *(A)* 2860 mg OTC-C and *(B)* 2860 mg OTC-LA.

4.4 DISCUSSION

Administration of OTC-C in the same dose as recommended for OTC-LA (20 mg/kg bwt), resulted in almost similar serum concentration-time curves, either injected IM or in the dewlap. The mean elimination half-life for OTC after IM injection was about 11 hours. This period agrees well with the 10 hours reported in cattle by Bywater (1982). The half-life for OTC after dewlap injection was considerably longer, about 18 hours. This difference indicates that during the elimination phase, continued slow absorption of OTC was still going on from the dewlap injection site.

In experiments on calves, Nouws (1982) reported a slight retard effect of OTC-LA after IM administration when comparing equal doses (18.1 mg OTC/kg bwt) of a conventional OTC preparation (Engemycin^{R} - 10%, containing 100 mg OTC/ml) and OTC-LA (Terramycin^{R}/LA, containing 200 mg OTC/ml). With both preparations the plasma concentrations remained above 0.5 µg OTC/ml for more than 48 hours.

In the present study the OTC serum concentrations after IM injection remained above 0.5 µg OTC/ml slightly longer after OTC-LA compared to OTC-C (52 vs 45 hours), and that was also the case after dewlap injection (62 vs 57 hours). The differences between AUCs for

the two preparations however, were not significant, whether injected IM or in the dewlap ($p > 0.10$). The present findings are in agreement with the results of Xia *et al.* (1983b). They were also unable to demonstrate any significant retard effect of OTC-LA compared to OTC-C after IM injection in cattle.

Although OTC-LA may offer a slight retard effect, the present study as well as those referred to, do not support the manufacturer's claim that therapeutic levels of OTC are maintained for 3 to 5 days after a single IM injection of 20 mg OTC-LA/kg bwt.

Luthman and Jacobsson (1982) ascribed the good clinical results after treatment with 20 mg OTC-LA/kg bwt, to the high dose administered and not to a sustained action of the preparation.

From experiments on calves (Nouws, 1982) and pigs (Xia *et al.*, 1983a), it was suggested that OTC-LA might exert a slight retard effect depending on the tissue damage at the site of injection.

After dewlap injection, the AUC from 0 to 96 hours, was only about 56% of that after IM injection. In addition to being less vascularised, the tissue damage, necrosis and oedema at the dewlap injection site, might have contributed to a slow absorption.

According to the manufacturer, a volume of 20 ml per injection site should not be exceeded in cattle for any of the OTC preparations. In this study the mean volumes used were 26.3 ml OTC-C and 15.3 ml OTC-LA. Although the recommended volume was exceeded for OTC-C, and the injected volume was almost double for that of OTC-LA, there was less tissue damage after dewlap injection of OTC-C compared with OTC-LA. IM injections of both OTC preparations were well tolerated.

Bergsjø (1976) concluded that injection in the dewlap might represent a valid alternative route to IM injection of aqueous penicillin. Injections in the dewlap have been employed by some veterinarians for the administration of drugs such as calcium borogluconate and quinuronium sulphate (Bergsjø, 1976).

The poor bioavailability of OTC after dewlap injection compared to IM injection makes this route unsuitable for administration of tetracyclines. The local tissue damage especially after dewlap injection of OTC-LA, also indicates that this route is not recommendabl for tetracyclines. While there was only a small difference between the histopathological changes produced by the two preparations after IM injection, there was a marked difference in disfavour of OTC-LA in the dewlap. The dewlap might be a suitable site to test

the tissue tolerance of various drug formulations.

After IM injection, no OTC residues were detected after the pre-slaughter time limit of four weeks. After dewlap injection, OTC residues were detectable, but low for both OTC preparations and did not exceed 0.1% of the administered doses. The kinetics of absorption and disappearance of OTC from the dewlap injection sites have not been investigated.

CHAPTER FIVE

A COMPARISON OF TWO OXYTETRACYCLINE PREPARATIONS (AQUACYCLINE^R AND TERRAMYCIN^R) WITH REGARD TO ABSORPTION CHARACTERISTICS, LOCAL TISSUE REACTIONS AND RESIDUES FOLLOWING DEWLAP INJECTIONS IN CALVES

5.1 INTRODUCTION

Large variations have been reported among injectable veterinary oxytetracycline (OTC) products with respect to bioavailability, peak serum drug levels and the extent of tissue reactions at the injection sites (Ziv, 1980; Nouws, 1982).

Aquacycline^R ("Rosco A/S", Denmark) is an OTC preparation which has recently been introduced in a 5 and 10% solution, with the claim that it offers several advantages. According to the manufacturers, the principle of Aquacycline^R is that the OTC in the form of of magnesium complex is dissolved in a minor amount of propylene glycol and polyvidon. The final products are to a considerable degree aqueous solutions with a pH of approximately 8. The stable solution of low viscosity should make it easy to inject with negligible pain and tissue irritation. It is further claimed to possess major advantages such as low toxicity, a large margin of safety and absorption

characteristics which maximise the antibacterial activity.

The aim of the present study was to examine some of the postulated advantages of the two Aquacycline^{R} formulations by comparing them with Terramycin^{R} - 100 ("Pfizer", USA), a 10% injectable OTC preparation which has been widely used in veterinary medicine. Following dewlap injection, the three preparations were compared with regard to OTC bioavailability and serum concentrations, as well as tissue reactions and residues at the site of injection.

5.2 MATERIALS AND METHODS

5.2.1 Animals

The trial included twelve mixed breed steers with a mean age of 10 months (range 8 - 13 months) and an average body weight of 146 kg (range 100 - 170 kg).

5.2.2 Drugs

The three oxytetracycline (OTC) formulations were:

OTC-A5 : Aquacycline^{R}-5 injectable solution containing 50 mg OTC/ml ("Rosco", Taastrup, Denmark).

OTC-A10 : Aquacycline^{R}-10 injectable solution containing 100 mg OTC/ml ("Rosco", Taastrup, Denmark).

OTC-C : Terramycin^{R} - 100 injectable solution containing 100 mg OTC/ml ("Pfizer International, Inc.", New York, USA).

They were administered as described in Table 8 (p.87).

5.2.3 Blood samples

Serum was obtained from ten ml blood samples drawn from the jugular vein as described in Chapter 4 (4.2.3., p. 64).

5.2.4 Registration of swelling

The swelling at the dewlap injection site was measured for a period of 4 weeks with the method described in Chapter 4 (4.2.4, p. 65).

5.2.5 Tissue samples

From each of the three groups, one calf was slaughtered 28 days and another 42 days after injection. The dewlap of each animal was excised around the site of injection and examined in the same way as described in Chapter 4 (4.2.5, p. 65).

5.2.6 OTC analyses

Quantitation of OTC in serum and tissue samples was performed by the microbiological assay method (Ch. 3.2, p. 28).

Table 8 Details of three groups of four calves injected in the dewlap with three different OTC preparations (20 mg OTC/kg bwt).

OTC preparation	Calf No.	Body weight, kg	OTC injected mg	Volume injected, ml
OTC-A5	055	120	2400	48.0
	057	116	2320	46.4
	064	100	2000	40.0
	068	107	2140	42.8
	$\bar{x}$	111	2215	44.3
OTC-A10	056	160	3200	32.0
	058	160	3200	32.0
	059	160	3200	32.0
	067	170	3400	34.0
	$\bar{x}$	163	3250	32.5
OTC-C	060	166	3320	33.2
	063	166	3320	33.2
	065	162	3240	32.4
	066	170	3400	34.0
	$\bar{x}$	166	3320	33.2

5.2.7 Histology

The dewlap specimens in 10% buffered formalin were processed for light microscopic examination (Ch. 4.2.7, p. 66).

4.2.8 Calculations

The areas under the OTC serum concentration - time curves (AUCs) were calculated by the trapezoidal method (Ch. 4.2.8, p. 66).

Student's t-test was used for statistical comparison between the mean values of the calculated AUCs (Durant, 1977).

The areas under the dewlap swelling-curves were calculated by the trapezoidal method and were estimated and examined for tissue irritation following injection of the three preparations.

5.3 RESULTS

5.3.1 OTC serum concentrations

Individual and mean OTC serum concentration curves for the three preparations are presented in Fig. 23, p. 91. Compared to the peak concentration of 1.8 µg OTC/ml after OTC-C, somewhat higher peak concentrations were recorded after the OTC-A preparations, being 2.1 and 2.6 µg OTC/ml following OTC-A10 and OTC-A5 respectively. Serum concentrations above 0.5 µg OTC/ml were reached

within 2 to 3 hours for all three preparations and maintained for 60 hours (range 53 - 63 hours) after OTC-C, 60 hours (range 56 - 65 hours) after OTC-A10 and 61 hours (range 60 - 64 hours) after OTC-A5. During this period, the AUCs for both the OTC-A preparations proved to be significantly larger than the corresponding AUC for OTC-C, ($p < 0.05$), while there was no significant difference between the AUCs for OTC-A5 and OTC-A10 ($p > 0.05$), see Appendix 2, p. 147.

5.3.2 Swelling

Between the calves there were rather large variations in the swelling at the site of injection, especially when injected with OTC-A5 (Fig. 24, p. 92). The mean recorded swelling during the first week following injection of OTC-A5 was 72% of the corresponding value after OTC-C ($p < 0.01$) while the swelling after OTC-A10 averaged 81% of that after OTC-C ($p < 0.05$), see Appendix 2, p. 147.

5.3.3 Necropsy

Macroscopic examination of dewlap injection sites 28 and 42 days after injection revealed areas of necrosis after all three OTC preparations (Fig. 25, p. 93; Fig. 26, p. 94 and Fig. 27, p. 95). The pathological changes were more pronounced in those calves which received OTC-C (Fig. 25, p. 93).

Microscopic examination of the dewlap injection sites 42 days after injection revealed a rather strong inflammatory reaction and thrombosis after OTC-C (Fig. 28, p. 96), a chronic inflammation with perivascular lymphocytic cuffings after OTC-A5 (Fig. 29, p. 97), while after OTC-A10 there was a milder inflammatory reaction with low grade of fibrosis (Fig. 30, p. 98).

5.3.4 OTC residues at the injection sites

Illumination of the dewlap injection sites with UV-light revealed yellow tetracycline fluorescence (Fig. 31, p. 99; Fig. 32, p. 100 and Fig. 33, p. 101). The fluorescence was less intense after OTC-A5 administration (Fig. 32, p. 100).

The total amounts of OTC residues were estimated to be 0.22 mg 28 days and 0.05 mg 42 days after injection of OTC-C in dewlap. The corresponding values for OTC-A10 were 0.32 and 0.03 mg, and for OTC-A5, 0.15 and 0.01 mg.

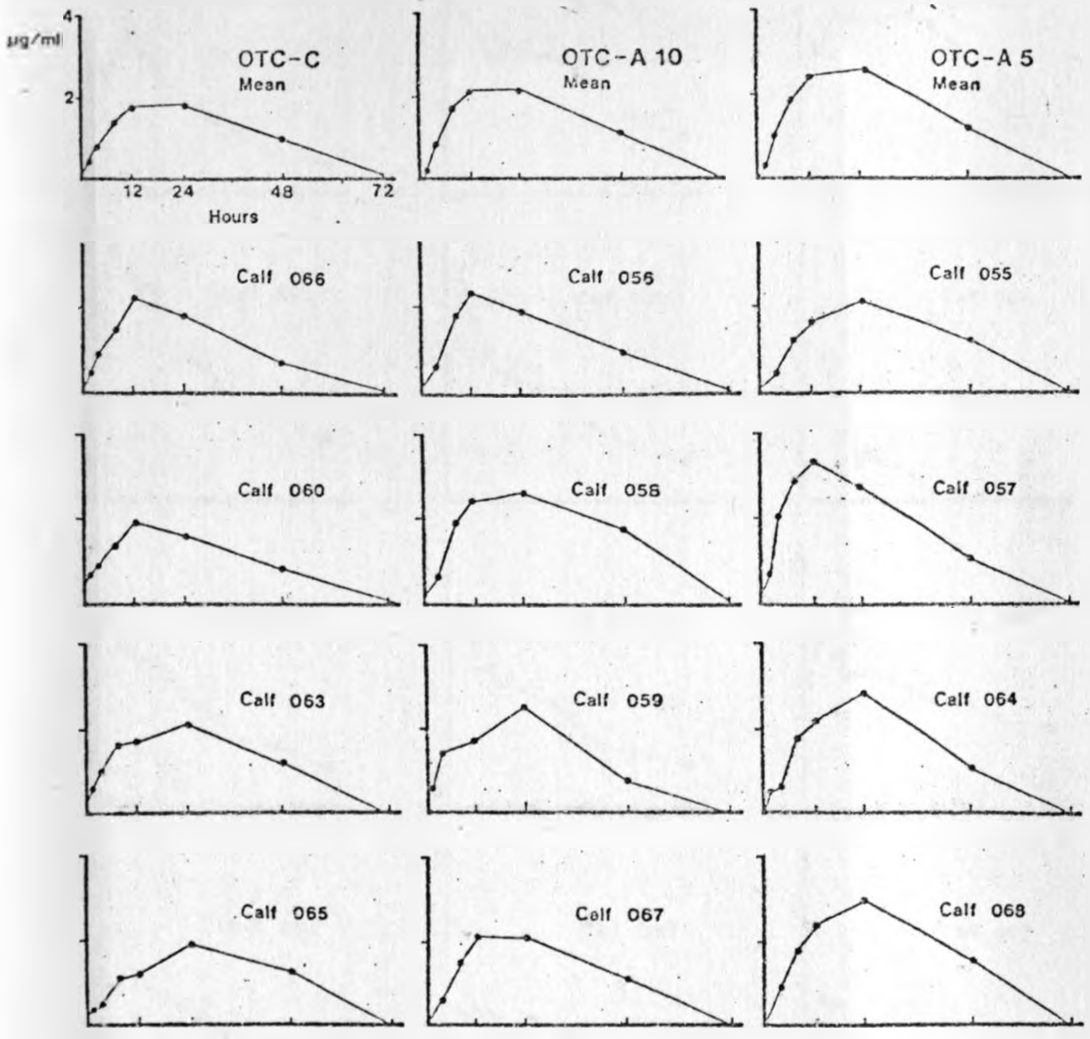


Fig. 23 Mean and individual OTC serum concentrations (µg/ml) in the three groups of four calves following dewlap injections (20 mg OTC/kg bwt) of OTC-C, OTC-A10 and OTC-A5.

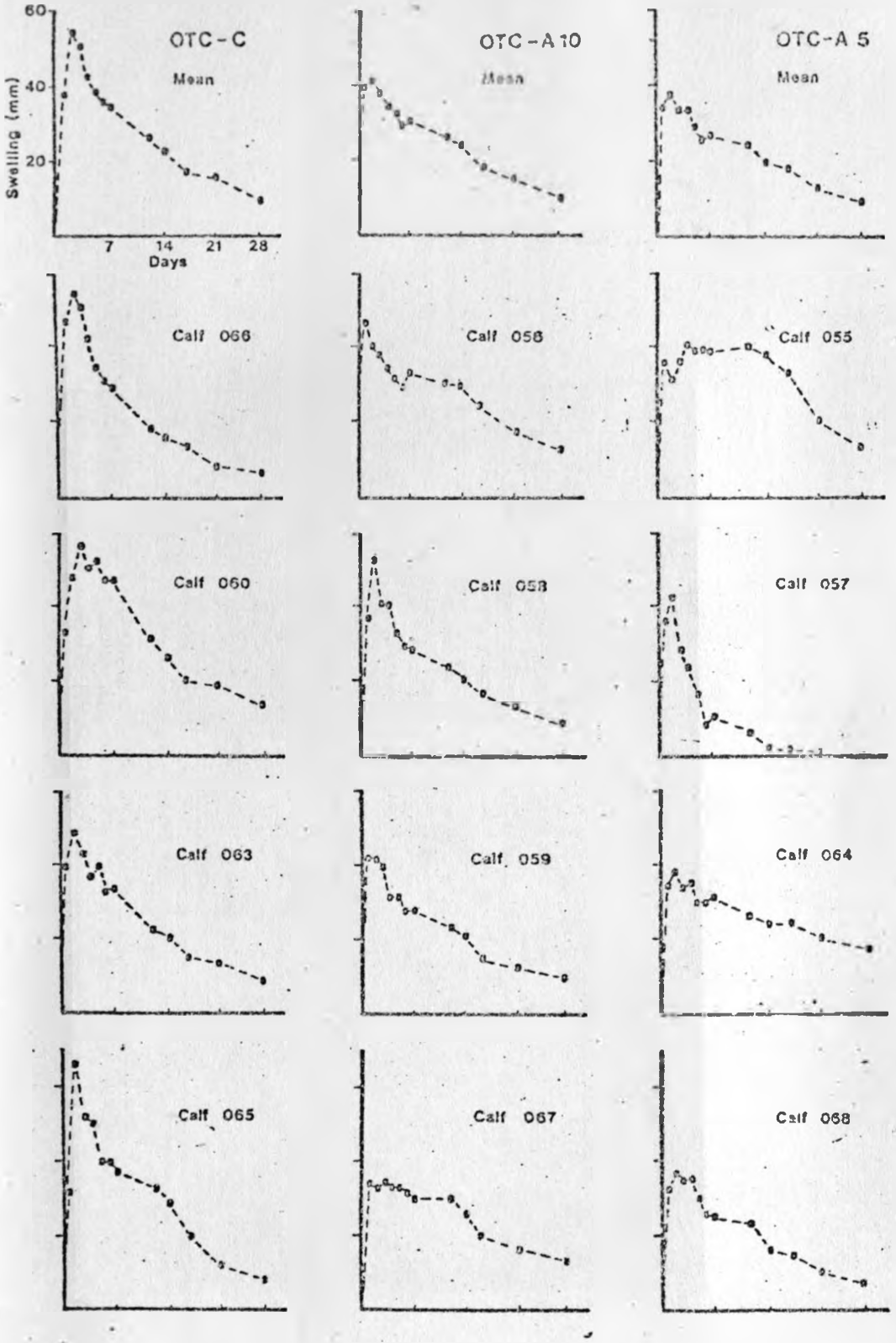


Fig. 24 Mean and individual swellings in the dewlaps in three groups of four calves following dewlap injections (20 mg OTC/kg bwt) of OTC-C, OTC-A10 and OTC-A5.

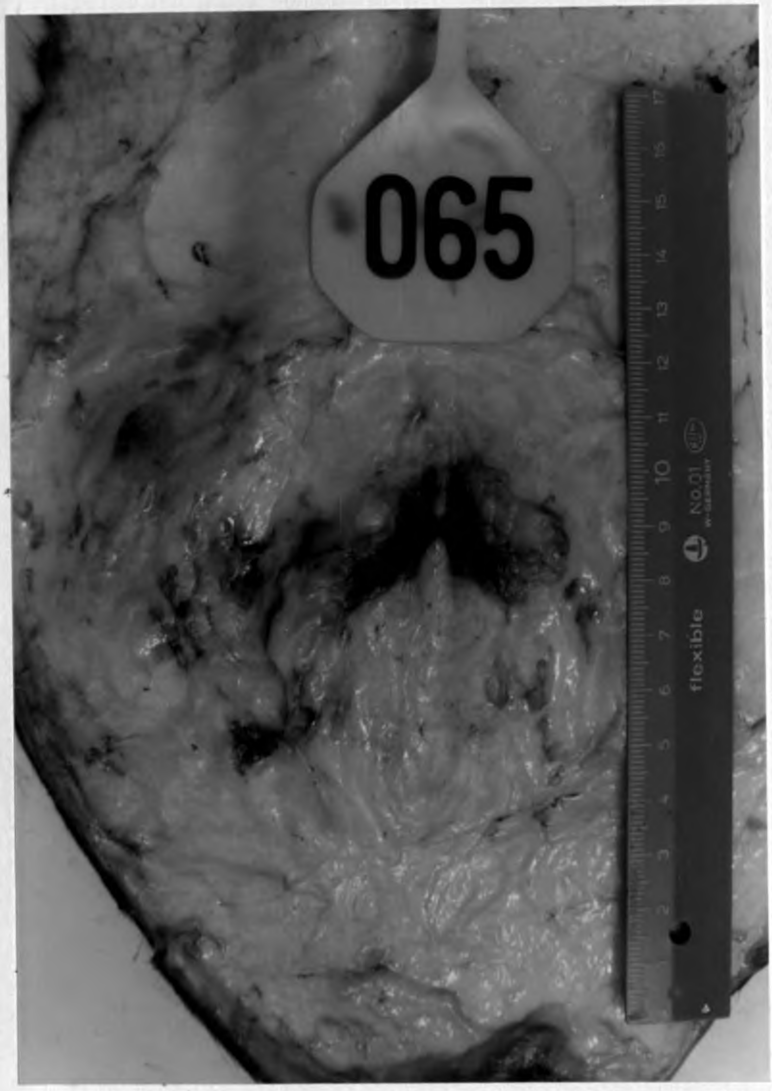


Fig. 25 Photograph of the dewlap injection site taken under ordinary light 42 days after injection of 3240 mg OTC-C (32.4 ml).

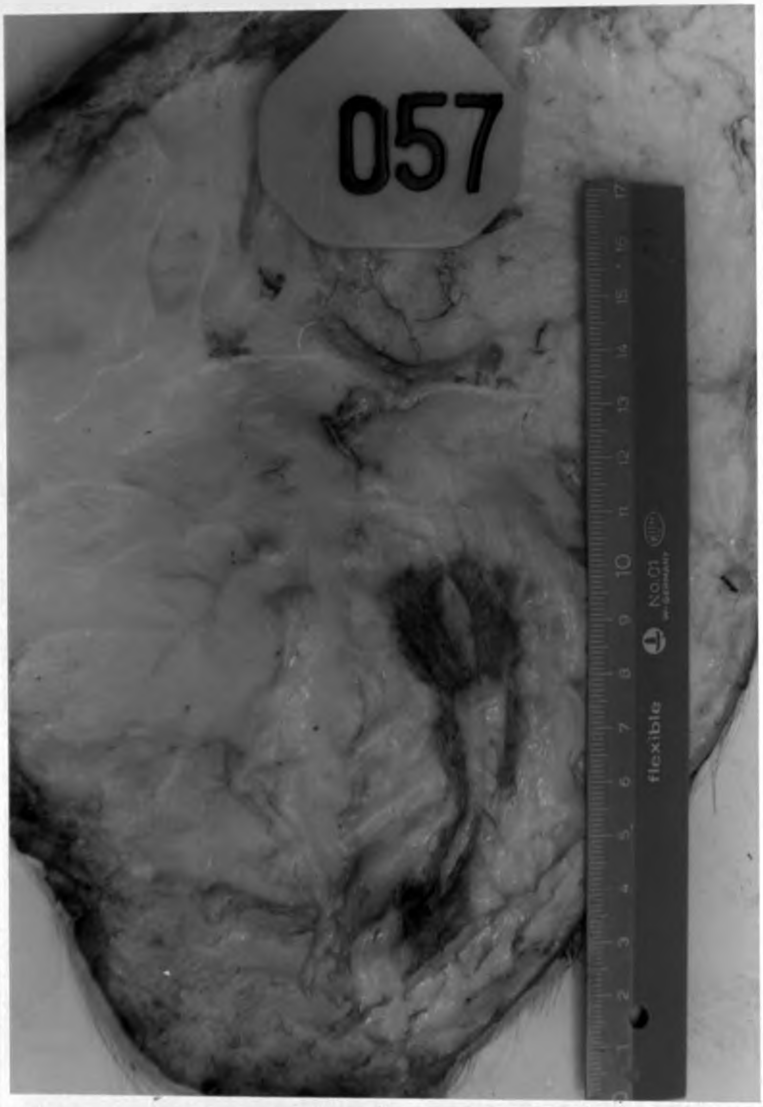


Fig. 26 Photograph of the dewlap injection site taken under ordinary light 42 days after injection of 2320 mg OTC-A5 (46.4 ml).

Fig. 27 Photograph of the dewlap injection site taken under ordinary light 42 days after injection of 3400 mg OTC-A10 (34.0 ml).

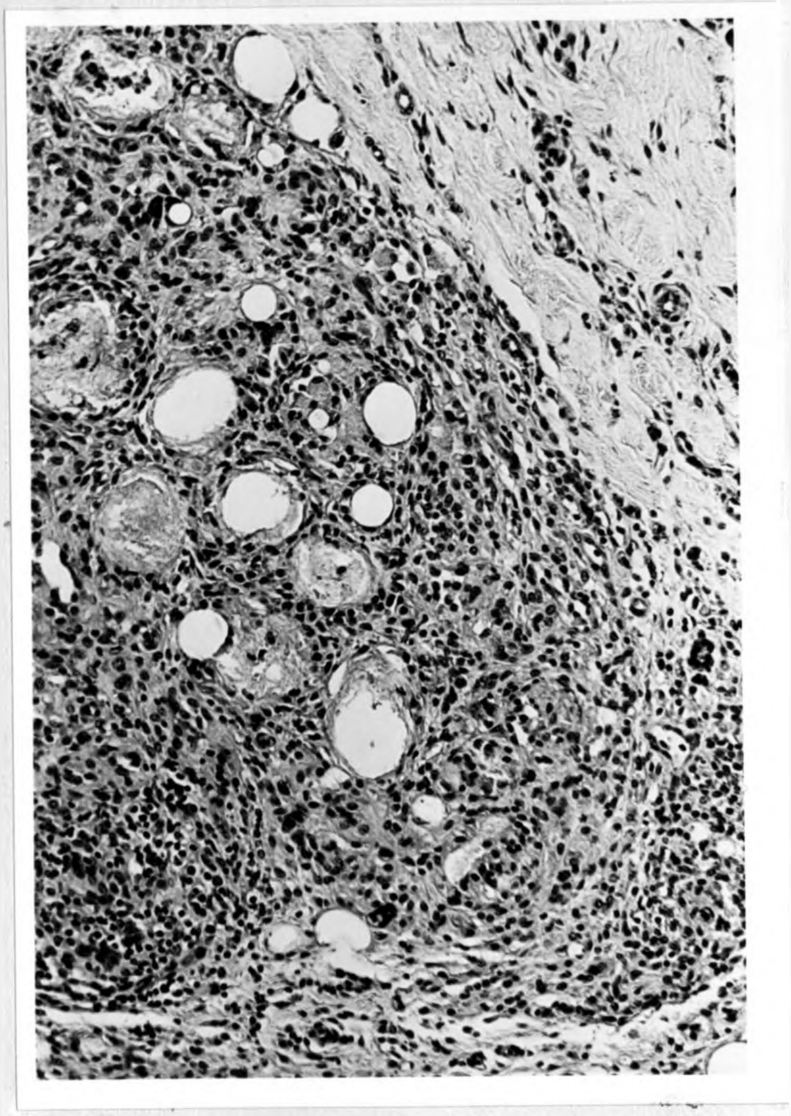


Fig. 28 Photograph of the histological section of the dewlap injection site 42 days after injection of 3240 mg OTC-C (32.4 ml) 170 x HE.

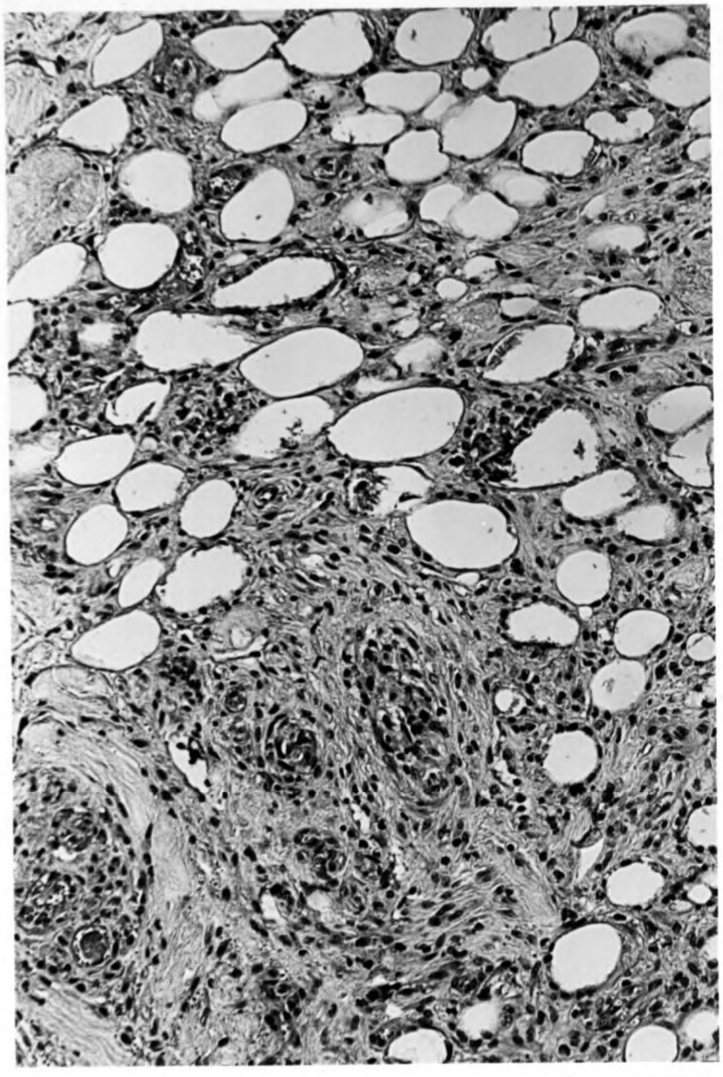


Fig. 29 Photograph of the histological section of the dewlap injection site 42 days after injection of 2320 mg OTC-A5 (46.4 ml) 170 x HE.

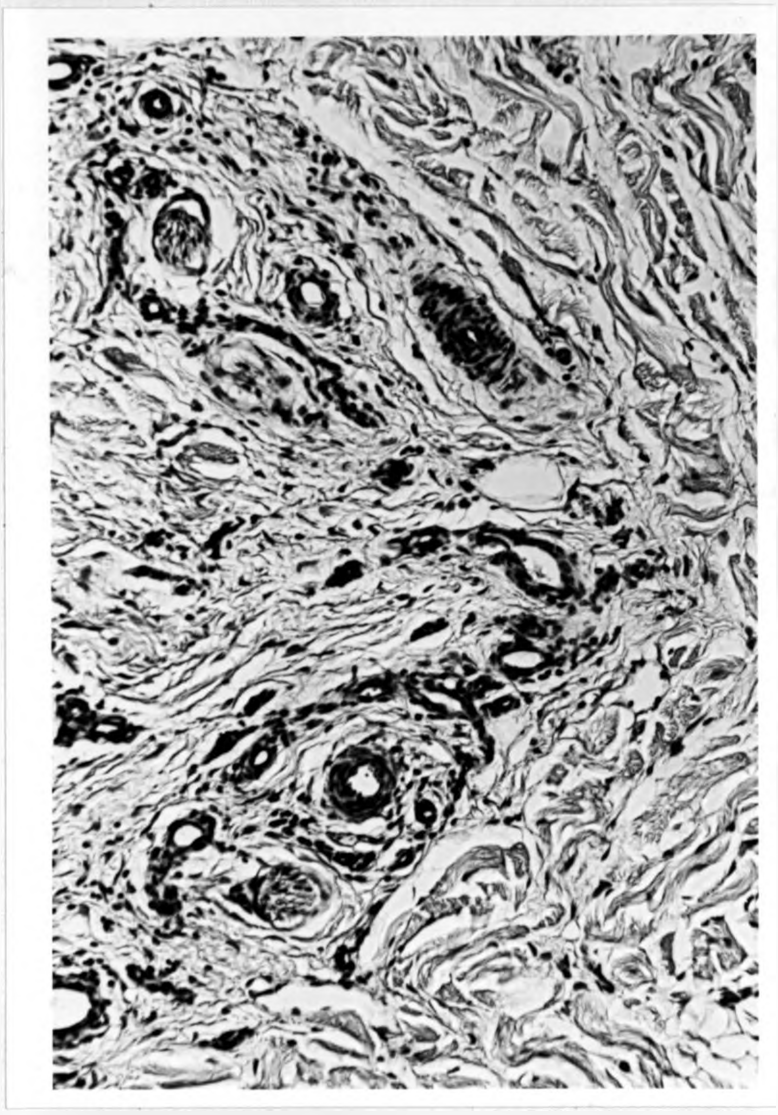


Fig. 30 Photograph of the histological section of the dewlap injection site 42 days after injection of 3400 mg OTC-A10 (34.0 ml) 170 x HE.

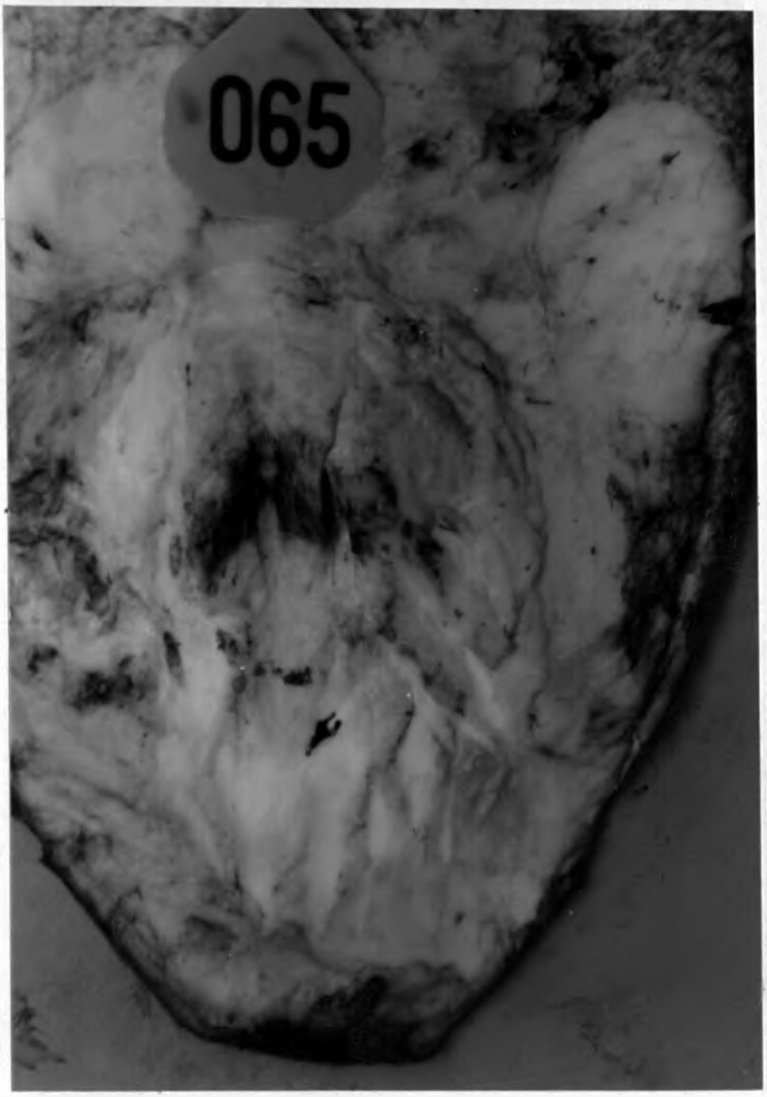


Fig. 31 Photograph of the dewlap injection site taken under UV-illumination 42 days after injection of 3240 mg OTC-C.

Fig. 32 Photograph of the dewlap injection site taken under UV-illumination 42 days after injection of 2320 mg OTC-A5.

Fig. 33 Photograph of the dewlap injection site taken under UV-illumination 42 days after injection of 3400 mg OTC-A10.

5.4 DISCUSSION

All three OTC preparations resulted in serum concentrations above 0.5 µg OTC/ml from about 2 hours till about 60 hours following injection. During this interval, OTC-A5 gave a 39% and OTC-A10 a 20% increase in the area under the serum concentration - time curves as compared to OTC-C ($p < 0.05$), see Appendix 2, p. 147. These findings support the claim that the OTC-A preparations offer advantages with respect to absorption characteristics.

At the injection site, detectable OTC residues outlasted a pre-slaughter time limit of four weeks, but they were low for all the three OTC preparations, and did not exceed 0.3 mg at 28 days and about 0.15 mg at 42 days.

Larger volumes of OTC-A5 were injected because of the lower OTC concentration in this preparation. The larger volumes did not cause an increased local tissue reaction, at least not with respect to the swelling. Since the average body weight of the calves injected with OTC-A5 was lower, the mean amounts of OTC injected in this group was only 2.2 g while it was 3.3 g for the other two groups. The smaller amount of OTC injected in the OTC-A5 group might partly explain the milder tissue reaction and the smaller residue after this preparation.

In response to a request for publications on Aquacycline^{R}, the manufacturer provided several published clinical studies with various OTC preparations, but not a single publication which specifically dealt with their own product. It is remarkable that the only available published information on Aquacycline^{R}, seems to be the information booklets of the manufacturer.

The present investigations give some support to the claims that Aquacycline^{R} offers advantages with regard to absorption characteristics and tissue tolerance.

CHAPTER SIX

PASSAGE OF OXYTETRACYCLINE THROUGH THE RUMINAL WALL AND ITS EFFECTS ON THE RUMINAL FERMENTATIVE ACTIVITY IN SHEEP

6.1 INTRODUCTION

There are many reports on gastrointestinal disturbances associated with tetracycline therapy. In ruminants, the clinical signs may consist of indigestion, anorexia and diarrhoea (Bywater, 1982; Huber, 1982).

In the rumen, both bacteria and protozoa are found in large numbers. These microorganisms provide the enzymes necessary to decompose otherwise indigestible plant material so that the ruminant animal, through the digestion of microorganisms, gains metabolic benefits from the ingestion of these food products. Fermentation processes within the reticulorumen provide volatile fatty acids (VFAs), proteins and vitamins. Acetic, propionic and butyric acids are the major VFAs formed by ruminal fermentation.

The present study was aimed at examining the absorption of OTC from the rumen as well as its passage into the rumen after parenteral administration.

It further aimed at examining the effects of OTC on the ruminal fermentative activity by using the three VFAs - acetic, propionic and butyric acids - as indicators.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Four sheep weighing about 60 kg each, were used. Two of them (Nos. 3 and 4) were fitted with permanent ruminal cannulae by a standard surgical technique before the start of the experiment. The animals were kept indoors and each was fed a daily diet of 1 kg of hay in the morning and 0.2 kg of concentrate ("MØllegaard", Denmark) in the afternoon, with free access to water.

6.2.2 Drugs

The oxytetracycline OTC preparation was:

Aquacycline^{R}-5 injectable solution containing 50 mg OTC/ml ("Rosco A/S", Taastrup, Denmark).

Sheep No. 1 received 10 mg OTC/kg bwt and sheep No. 2, 20 mg OTC/kg bwt by IV injection, while sheep Nos. 3 and 4 were administered 3 mg OTC/kg bwt into the rumen (IR) via the cannulae. The drug was given before the sheep were fed with hay.

6.2.3 Blood samples

Ten ml blood samples were drawn from the jugular vein into heparinised vacutainers (Venoject^{R}, "Terumo Corporation", Japan) before and at the following time intervals after drug administration:

After IV injection (Sheep Nos. 1 and 2):

10, 20, 30, 45 minutes; and 1, 2, 3, 4, 6 and 24 hours.

After IR administration (sheep Nos. 3 and 4):

1, 2, 3, 4, 6, 8, 26, 30, 50, 54 and 72 hours.

The blood was centrifuged for 10 minutes at 5400 g and the separated plasma was kept at -20^{o}C until analysed.

6.2.4 Ruminal fluid samples

For OTC determination, 5 ml ruminal fluid samples were collected at the same time intervals as blood and kept at -20^{o}C until analysed. From sheep Nos. 1 and 2 the ruminal fluid samples were collected through a stomach tube and from sheep Nos. 3 and 4 through the ruminal cannula.

For VFA determination, a further 5 ml ruminal fluid samples were collected from sheep Nos. 3 and 4 before and at the following time intervals after drug administration:

2, 6, 26, 30, 50 and 54 hours.

The ruminal fluid samples were strained through a sieve for the removal of particulate matter. To stop the fermentation, 0.5 ml of 25% phosphoric acid was added to each tube containing 2.5 ml aliquots and allowed to stand for 30 minutes. The tubes were centrifuged for 10 minutes at 2000 g. The supernatants obtained were refrigerated for subsequent VFA analysis.

6.2.5 Analyses

OTC concentrations in the plasma samples were determined by both the microbiological assay (Ch. 3.3, p. 35) and the HPLC method (Ch. 3.4, p. 40), while OTC in the ruminal fluid was quantitated by only the microbiological assay method.

VFA concentrations were quantitated by the gas-liquid chromatography (Fractorap 4200 "Carlo Erba", Strumazione, Italy) on 0.6 µl aliquots of the supernatants according to the method described by Baldwin *et al.*, 1982, with 2 - ethyl butyrate as an internal standard. VFAs were analysed on columns packed with GP 10% SP - 1000/1% ($v/_v$) H_3PO_4 on 100/120 Chromosorb^R W AW ("Supelco", S.A., Switzerland). The nitrogen flow rate was 40 ml/min., the injector and detector temperatures were 200^oC and the oven temperature was 125^oC. The internal standard concentration was kept constant (5 mmol) and

the standards of the three VFAs were injected in 0.5, 5 and 50 mmol concentrations.

6.2.6 Calculations

The concentrations of OTC in plasma and ruminal fluid samples were calculated as described in Chapter 3 (3.2, p. 28).

The unknown amount of each VFA in a sample was calculated based on the following equation:

$$C_a = \frac{A_a}{A_{is}} \times C_{is}$$

where:

C_a = mmol concentration of the acid: acetic acid, propionic acid or butyric acid

A_a = peak area of the acid: acetic acid, propionic acid or butyric acid

A_{is} = peak area of the internal standard

C_{is} = concentration of the internal standard

Total VFA was the sum of the three acids.

6.3 RESULTS

6.3.1 OTC plasma concentrations

The OTC plasma concentration curves following IV administration of 10 and 20 mg OTC/kg bwt are shown in Fig. 34, p. 110. There was a good correlation

between the results obtained with the HPLC and the microbiological assay. Two hours after OTC administration of 10 and 20 mg/kg bwt, the plasma concentrations were about 9 and 20 µg OTC/ml, and after 6 hours the corresponding concentrations were about 3 and 5 µg OTC/ml. After 24 hours, the plasma levels had fallen below 0.5 µg OTC/ml in both animals.

No OTC could be detected in plasma after IR administration.

6.3.2 OTC concentrations in ruminal fluid

No OTC could be detected in the ruminal fluid following IV injection.

One hour after IR administration, the concentration of OTC was about 15.8 µg OTC/ml ruminal content. The corresponding values after 2, 6 and 30 hours were 9.6, 4.7 and 1.1 µg OTC/ml.

OTC could not be detected after 50 hours (Table 9, p. 111).

6.3.3 VFA production

Intraruminal administration of OTC had only a modest effect on the VFA production. There were tendencies towards a reduction in the total VFA and an increase in the acetic/propionic acid ratio.

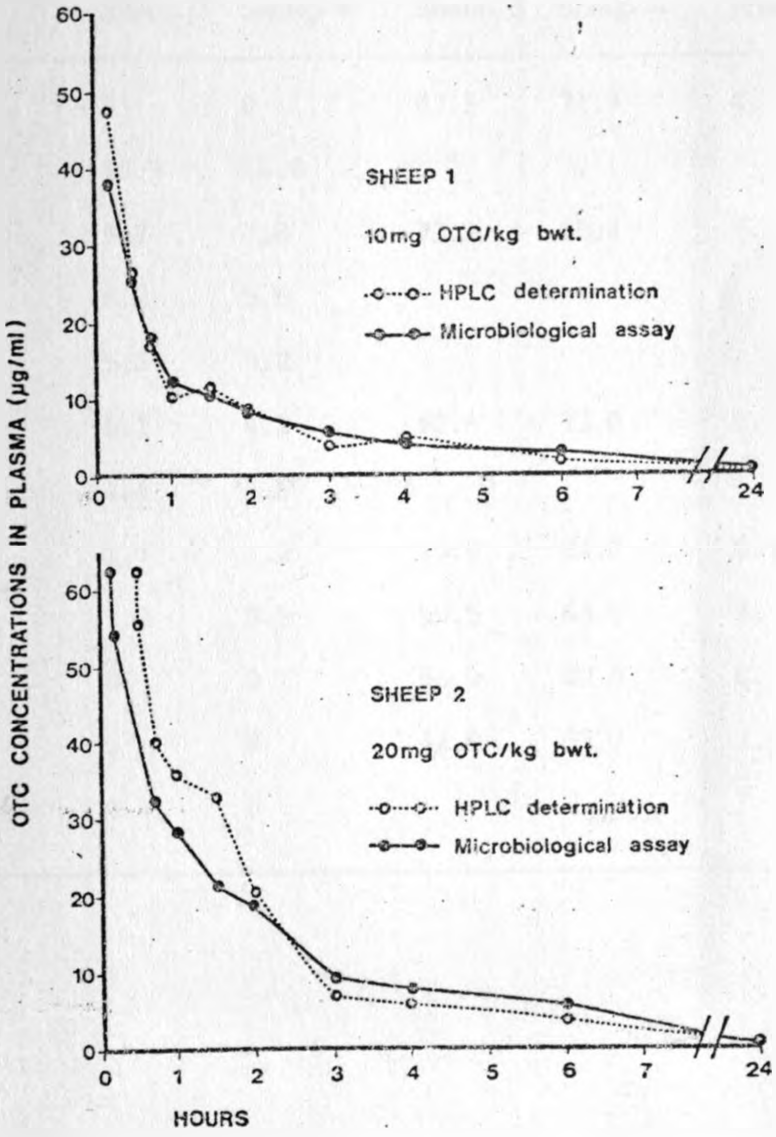


Fig. 34 OTC concentrations in plasma of two sheep after IV injection of 10 or 20 mg OTC/kg bwt.

Table 9 Concentrations of OTC in the rumen of two sheep, VFA production and the ratio between acetic and propionic acid, following IR administration of 3 mg OTC/kg bwt.

Time interval, hours	OTC µg/ml		VFA mmol		Acetic/propionic acid	
	Sheep 3	Sheep 4	Sheep 3	Sheep 4	Sheep 3	Sheep 4
0	0	0	67.3	71.9	4.7	4.8
1	15.5	16.0				
2	9.3	7.8	73.5	80.4	4.0	4.2
3	6.2	5.8				
4	5.8	5.2				
6	5.2	4.2	62.6	73.0	6.0	5.6
8	4.4	3.6				
26	1.5	1.2	75.5	64.0	5.1	5.6
30	1.2	0.9	59.5	64.8	5.9	6.9
50	0	0	56.0	49.0	6.1	5.4
54	0	0	64.0	52.7	7.5	6.7
72	0	0				

6.4 DISCUSSION

In the present study, OTC could not be detected in the ruminal fluid following IV injection and neither was it possible to detect OTC in plasma after IR administration.

The reticuloruminal epithelium is permeable to lipid soluble compounds and as a lipid soluble compound OTC could be expected to pass, similar to drugs such as antipyrine, sulphanilamide and salicylate (Stowe, 1967; Jenkins, 1982). There are, however, several factors which may modify or interfere with drug passage through the ruminal wall, such as binding to ingesta in the rumen, the dilution factor and the small ratio of surface area to volume of contents (Stowe, 1967; Jenkins, 1982).

The observed tendencies towards a reduction in total VFA and an increase ratio between acetic and propionic acid after IR administration of OTC in sheep, agree with the results obtained in cattle (Behravesh *et al.*, 1982; Jenkins, 1982) and *in vitro* experiments (O'Connor *et al.*, 1970; Baldwin *et al.*, 1982) on the effect of tetracyclines on ruminal fermentative activity.

Assuming that about 6 - 12 kg contents are in the reticulorumen of a 60 kg sheep, an even distribution of the administered dose, 180 mg, gives a theoretical concentration of 15 - 30 µg OTC/ml. The concentration

found after one hour was about 16 µg OTC/ml. At this level a bacteriostatic effect of OTC is to be expected. Besides bacteria, protozoa play an important role in the fermentative process in the rumen. A possible explanation for the moderate effect of OTC on the ruminal fermentative activity, is that protozoa are relatively resistant to tetracyclines (Huber, 1982).

CHAPTER SEVEN

A STUDY ON THE POTENTIAL OF TETRACYCLINE ADMINISTRATION TO INDUCE CHANGES IN THE FOETAL EYE WHEN GIVEN SYSTEMICALLY TO PREGNANT RATS AND RABBITS

7.1 INTRODUCTION

The systemic use of tetracyclines during mammalian pregnancies may cause several side effects in the offspring. Since the initial report by Shwachman and Schuster (1956), it has been widely recognized that tetracyclines may be deposited during tooth formation and cause discolouration and dental hypoplasia. Tetracyclines are also deposited in calcifying areas in bone and in keratinized tissues. They may, at least reversibly interfere with bone growth. A 40% depression of bone growth, as determined by measurement of fibulas, has been reported in premature infants treated with tetracyclines (Cohlan *et al.*, 1963).

In preliminary communications, Harley *et al.*, 1964; 1965), suspected that aromatic drugs like tetracyclines might be responsible for congenital cataracts, but they stressed that there was no firm evidence. More recently, Krejči *et al.*, 1980, reported corneal discolouration and lens opacities in newborn rats as a complication of intramuscular use of tetracycline in pregnancy. This

alarming report initiated the follow-up studies presented in this Chapter.

Krejči *et al.* (1980) checked the eyes on a slit lamp and examined them histologically in UV-light for tetracycline fluorescence. These methods were also used in the present study on the potential of tetracycline to be deposited and induce changes in the foetal eye. In addition to rats, rabbits were used, and it was further found relevant to supplement the methods for detecting tetracycline deposits with autoradiographic studies with tritium-labelled tetracycline.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Five pregnant Wistar rats ("Møllegaard", Denmark) with an average body weight of 200 g and five pregnant chinchilla rabbits weighing approximately 4 kg each, were used. They were housed individually in cages at 20°C and 40-60% relative humidity. The rats were fed with a commercial pelleted diet ("Møllecentralen A/S", P. Larsen and Co., Oslo, Norway), and the rabbits with a pellet breeding diet ("Ewos", Södertälje, Sweden). Water was offered *ad libitum*. The light-darkness cycle was 12-12 hours.

7.2.2 Drug administration and pregnancies

Tetracycline (TC) powder, "A/S Apothekernes Laboratorium", Oslo, Norway, was dissolved in saline to a 10% ($w/_v$) solution. Two rats were given 20 mg TC/kg bwt daily by IM injection, starting 9 days ante partum and continuing until term. Three rabbits received the same daily dose by IM injection from 18 days ante partum until term.

The five pregnancies resulted in 8 newborn rats and 23 newborn rabbits which were weighed and the sex determined before killing by decapitation. The sacrifice of the offsprings was performed at 1,6,10 and 14 days post partum. Four newborn rats and four newborn rabbits, not exposed to TC *in utero*, served as controls.

Tritium-labelled tetracycline (Net - 141 tetracycline (7 - ^{3}H(N) with specific activity of 635.4 mCi/mmol and radiochemical purity >97% was obtained from "New England Nuclear", Boston, MA, USA. Decomposition on storage for 3 months was less than 1% at -10°C. Before use, ^{3}H - tetracycline (^{3}H - TC) was dissolved in saline.

Two rats received 0.14 mg ^{3}H-TC (0.2 mCi) by IV injection 11 days ante partum and they were euthanised with diethyl ether on the 20th day of gestation. Another rat received the same dose on the 20th day of

gestation and was euthanised 20 minutes after injection.

One rabbit was given 1.75 mg ^{3}H-TC (2.5 mCi) by IV injection 18 days ante partum and was euthanised with pentobarbitone on the 28th day of gestation. Another rabbit received the same dose on the 28th day of gestation and was euthanised 20 minutes thereafter.

7.2.3 Eye processing and examination

The eyes of each offspring from the mothers given unlabelled TC, were enucleated carefully and examined for visible TC deposits with a slit lamp. Thereafter, one eye was fixed in unbuffered 10% formalin for 48 - 72 hours until processed, and the other eye was frozen in freon prior to cryostat sectioning.

The eyes in 10% formalin were rinsed in cold tap water several times before clipping off any loose tissue around the eye balls. They were then dehydrated in 60% alcohol overnight. The central sagittal portions of the eyes were cut and placed in 80% alcohol for 8 hours, after which they were kept in 96% alcohol overnight. They were then rinsed in fresh 96% alcohol before being kept in absolute alcohol for about 12 hours. Thereafter, they were cleared twice for two hours in chloroform, and they were then embedded in paraffin wax for which sections of 5 - 8 µ thickness were cut. Sections for

light microscopy examination were stained with haematoxylin and eosin, while unstained eye sections were prepared for fluorescence microscopy. The eyes frozen in freon were exposed to liquid nitrogen (-196^{o}C) and sagittal sections (10 µ) of the central portions were made in a cryostat.

Microscopic examination - The stained paraffin sections were examined by light microscopy. Both the unstained paraffin embedded and the cryostat sections of the eyes were mounted in polyvinyl alcohol solution of pH 7.4 and examined by a Leitz Ploemopak^{R} fluorescence system, fitted with HBO 200 watts UV-light source and using filter block B_2 (exciting filter BP 350 - 410, beam splitting mirror RKP 455, suppression filter LP 455) and filter block D (exciting filter BP 355 - 425, beam splitting mirror RKP 455, suppression filter LP 460). A rat incisor with tetracycline deposits in the dentine was used as a positive control.

7.2.4 Autoradiography

The pregnant uteri and the maternal kidneys and livers of the rats and rabbits were removed immediately after sacrifice and underwent the same autoradiographic procedures described in Chapter 3 (3.8, p. 56).

7.3 RESULTS AND DISCUSSION

Following daily administration of TC to pregnant rats, commencing at various times between 6 - 16 days ante partum and continuing until term, Krejči *et al.* (1980) reported yellow discolouration in the eyes of the newborns and fluorescence when the cryostat eye sections were examined in UV-light. The total TC doses administered ranged from 37.5 to 70.5 mg, and they concluded that the most critical period seems to be 6 - 16 days ante partum.

In the present trial, the rats received 20 mg TC/kg bwt daily from 9 days ante partum until term, resulting in a total dose of 38 mg TC. The rabbits were given the same daily dosages from 18 days ante partum, which added up to a total of 1440 mg TC. This interval includes the most critical period of the eye development in rabbits.

In contrast to what was reported by Krejči *et al.* (1980), the present investigations did not reveal any TC deposits or changes in the eyes of the newborns. The findings were negative both when examined for visible discolouration on the slit lamp and for TC fluorescence in UV-light. No morphological abnormalities were detected when the stained eye sections were examined with light microscopy (Fig. 35, p. 121). A light

green fluorescence was observed in the retinal pigment epithelium and the photoreceptor layer, but this occurred in the control eyes as well, and was considered to represent normal retinal autofluorescence.

Furthermore, the autoradiographic studies with ^{3}H-Tc did not reveal any detectable ^{3}H - activity in the foetal eyes of rats or rabbits, whether administered in the mid-pregnancies or just before term. The embryos from the mothers which received ^{3}H-TC in the mid pregnancy had only very faint traces of ^{3}H - activity in the bone structures when killed at term. Compared to the maternal tissues, the concentration of radio-activity was low in the foetuses sacrificed twenty minutes after ^{3}H-TC administration, and the foetal eyes showed no detectable radioactivity (Figs. 36, p. 122 and 37, p. 123).

The present investigations failed to verify the findings of Krejči *et al.* (1980) that adminsitration of TC during pregnancy may result in the deposition of TC in the foetal eye causing discolouration and lens opacities. Since their publication in 1980, there seems to have been no other publication, either by their group or other investigators, which has confirmed their findings.

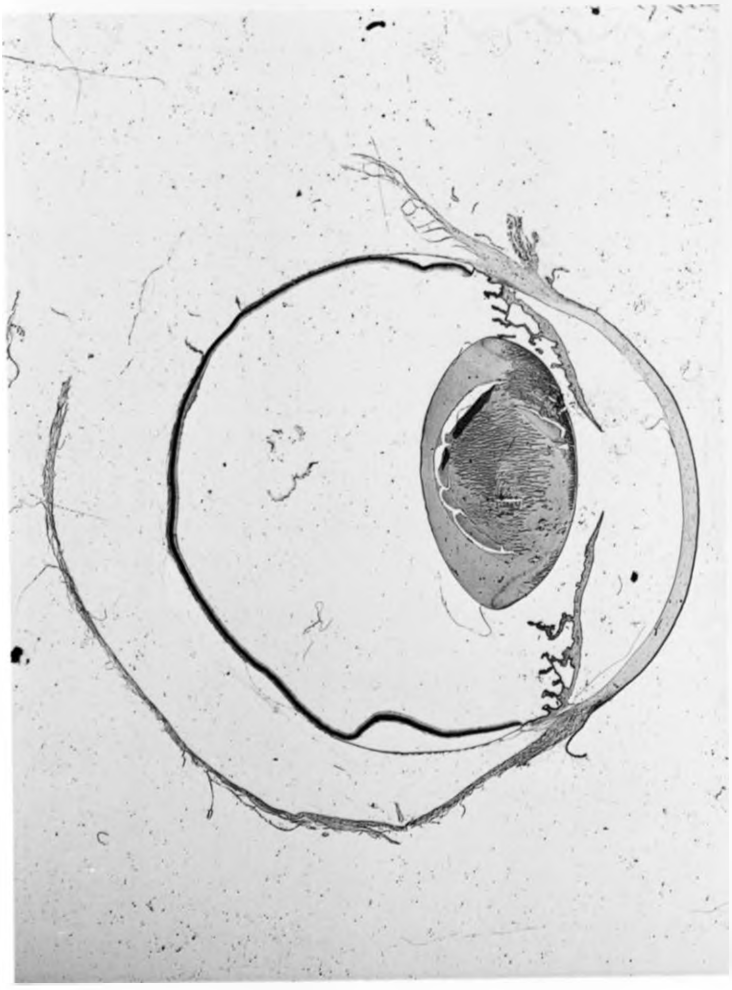
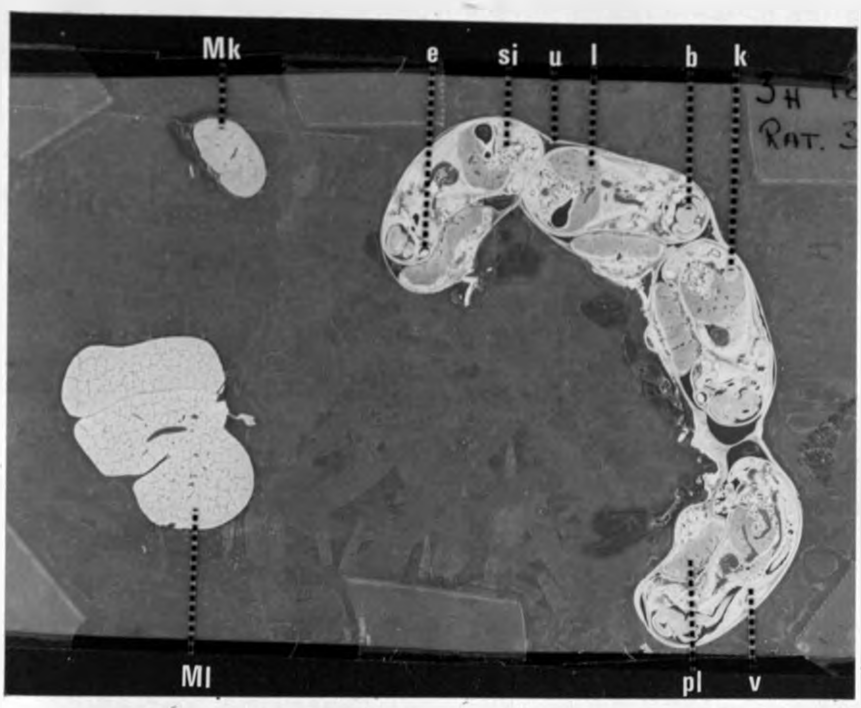


Fig. 35 Photograph of the histological section of the eye from a new-born rabbit exposed to TC *in utero* 10 x HE.



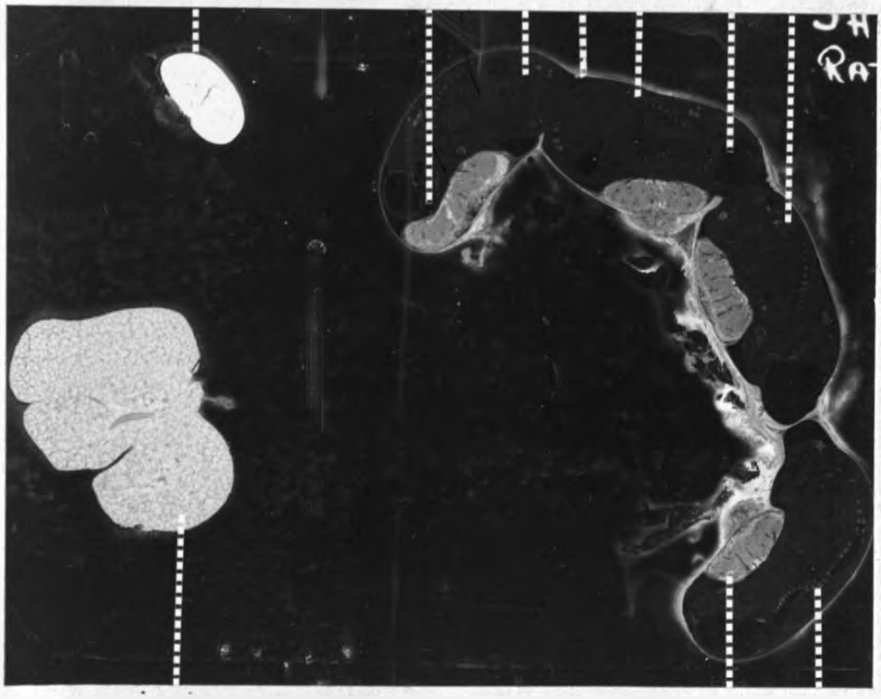
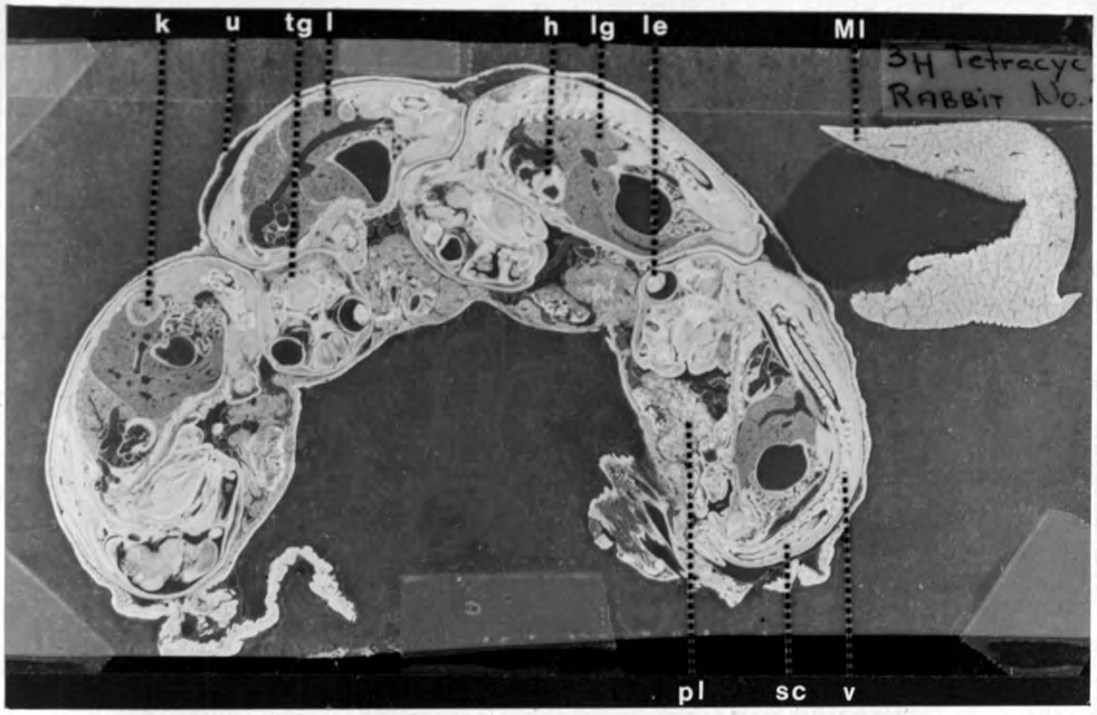
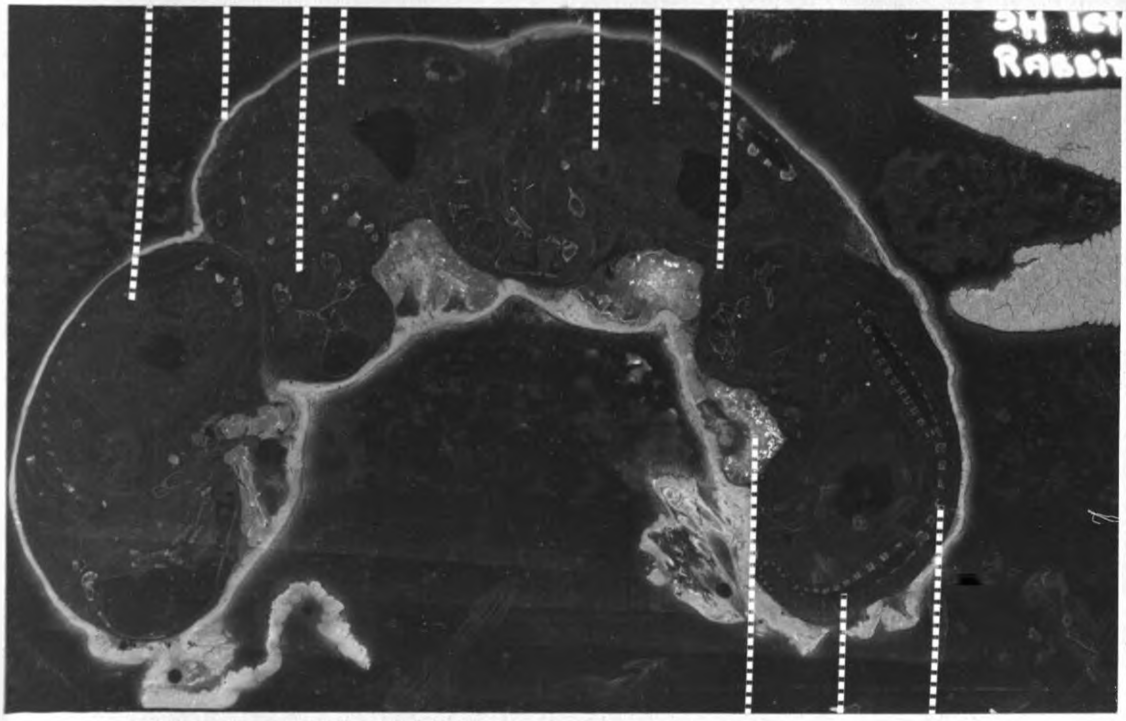


Fig. 36 Photograph of the unstained section *(A)* and the corresponding autoradiogram *(B)* of 20-day old rat embryos, maternal kidney and liver, 20 minutes after injection of 0.14 mg ^{3}H-tetracycline (0.2 mCi). Exposure time for the autoradiogram 3 months.

si = small intestine *v* = vertebral column *e* = eye *u* = uterus
ml = maternal liver *mk* = maternal kidney *l* = liver *b* = brain
k = kidney *pl* = placenta



A



B

Fig. 37 Photograph of the unstained section *(A)* and the corresponding autoradiogram *(B)* of 28-day old rabbit embryos and maternal liver twenty minutes after injection of 1.75 mg ^{3}H-tetracycline (2.5 mCi). Exposure time for the autoradiogram 3 months.

u = uterine wall
v = vertebral column
k = kidney
sc = spinal cord
ml = maternal liver
pl = placenta
tg = tongue
le = lens
lg = lung
h = heart

CHAPTER EIGHT
GENERAL CONCLUSIONS

1. In pilot experiments on the pharmacokinetics of oxytetracycline in rats and rabbits, a microbiological assay for tetracycline determination was found to be simple and to give reproducible and accurate results, with a lowest detectable concentration of about 0.1 µg oxytetracycline per ml serum or g tissue.

2. The oxytetracycline inhibition zones of the microbiological assay were reduced when certain cations (Al^{3+} and Mg^{2+}) were added to the medium, while the addition of ethylenediamine tetraacetate increased the inhibition zones and thereby apparently enhanced the sensitivity of the assay.

3. A comparison of the microbiological assay with a high pressure liquid chromatography method for the quantitation of oxytetracycline, revealed an excellent correlation between the two methods at concentrations between 1.0 and 40.0 µg oxytetracycline per ml plasma ($r = 0.99$).

4. Tetracycline fluorescence induced by UV-light

exposure proved suitable as a semiquantitative method for the detection of oxytetracycline in biological specimens.

5. Whole-body autoradiography with isotope-labelled tetracycline was also found to represent a useful method for studies on tetracycline pharmacokinetics.

6. In experiments with calves, no significant difference in the oxytetracycline serum concentrations could be demonstrated when a "conventional" preparation (Terramycin^{R}-100) was administered in the same dosage as recommended for the "long-acting" formulation, Terramycin^{R}/LA. These results do not support the claim that therapeutic levels of oxytetracycline are maintained for 3 to 5 days after a single injection of Terramycin^{R}/LA. Neither was this preparation found to offer advantages with regard to tissue damage and residues at the site of injection.

7. Injection of oxytetracycline in the dewlap proved not to be a useful alternative to the intramuscular route since, as indicated by the areas under the serum concentration curves, the bioavailability of this drug after dewlap injection was only about half of that obtained after intramuscular administration

The dewlap may, however, be a suitable site for tests designed to evaluate tissue tolerance to various drug formulations.

8. When a recently introduced oxytetracycline preparation, Aquacycline^{R}, was tested against Terramycin^{R}-100 in calves, Aquacycline^{R} resulted in about 30% greater area under the oxytetracycline serum concentration - time curve and about 25% less swelling at the site of injection. Accordingly, this preparation offered significant advantages both with respect to absorption characteristics and tissue tolerance.

9. There was apparently no passage of oxytetracycline through the ruminal wall, since oxytetracycline could not be detected in the ruminal fluid of sheep following intravenous injection, and neither was it possible to detect the drug in plasma after intraruminal administration.

10. The ruminal fermentation in sheep, as indicated by the production of volatile fatty acids, was only insignificantly affected by intraruminal administration of oxytetracycline.

11. The present investigations failed to confirm the alarming findings of a recently published study in rats, that administration of tetracycline during pregnancy may result in the deposition of tetracycline in foetal eye causing corneal discolouration and lens opacities.

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APPENDICES

Appendix 1 Areas under the OTC serum concentration - time curves (0 - 96 hours)

Preparation	Calf No.	Areas under the curves	
		IM	dewlap
OTC-C	1	115.4	78.2
	2	161.3	85.2
	3	145.2	70.8
	4	138.5	76.9
	$\bar{x}$	140.1	77.8
OTC-LA	5	152.4	101.2
	6	189.5	73.9
	7	130.3	80.7
	8	126.9	78.1
	$\bar{x}$	149.8	83.5

Appendix 2 Areas under the OTC serum concentration - time curves and dewlap swelling - time curves

Preparation	Calf No.	Serum curves, 0 - 60 hours	Swelling curves, 0 - 7 days
OTC-A5	055	83.3	231.5
	057	115.4	158.0
	064	100.3	216.5
	068	111.0	208.5
	$\bar{x}$	102.5	203.6
OTC-A10	056	80.8	235.5
	058	103.3	243.0
	059	87.0	227.0
	067	83.7	213.0
	$\bar{x}$	88.7	229.6
OTC-C	060	70.4	309.5
	063	82.9	252.5
	065	75.5	297.5
	066	66.4	271.5
	$\bar{x}$	73.8	282.8