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STUDIES ON SHEEP DIP BACTERIOSTATIC
AGENTS

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by
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DEDICATION

This thesis is dedicated to the
memory of the late

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ABSTRACT

In this study of bacteriostatic agents used in sheep dips, the results of earlier relevant work were first reviewed. The literature relating to the microbiology of fleece wool, the chemicals that have been used in the control of arthropod ectoparasites of sheep, and the micro-organisms of the fleece that cause problems with plunge sheep dipping, and the in vitro methods of testing the sensitivity of bacteria to chemicals was studied.

The seven bacteriostatic compounds selected for study were: lauryldimethylbenzylammonium chloride, a mixture of alkyl-, tolyl-, and methyl- trimethylammonium chlorides, 2-hydroxypropylmethane-thiosulphonate, 4-chloro-2-cyclopentylphenol, 2,2'-dihydroxy-3,5,6,3',5',6'-hexachlorodiphenylmethane, 3,4,4'-trichlorocarbanilide, and an arsenical preparation containing sodium arsenite. They were evaluated against the following indicator organisms; Escherichia coli (Migula) Castellani and Chalmers 1919, a sulphur reducing strain of E. coli, Pseudomonas aeruginosa (Schroeter) Migula 1919, Erysipelothrix rhusiopathiae (Migula) Buchanan 1918, and Dermatophilus congolensis van Saceghem 1915, emend. 1916, 1934.

The laboratory methods used to assess the activity of the compounds were; a broth dilution turbidimetric and agar subculture technique, an agar diffusion technique, a manometric technique, and a gradient plate procedure. A field trial was undertaken to relate the laboratory results to the practical sheep dipping situation.

The quaternary ammonium compounds tested showed the greatest antibacterial activity, and their use as dip bacteriostats can be recommended. All the other compounds, except 3,4,4'-trichlorocarbonylilide, have possible applications for the control of specific microbiological problems of sheep dipping or in fleece wool.

Some observations are made concerning the phenomenon of hormesis as shown in agar diffusion plates. Comparisons have been drawn between the methods used in this study and assessment of their value has been made. An agar diffusion method is suggested as a standard test for sheep dip bacteriostatic agents. Finally some recommendations for further research work have been made.

CONTENTS

	PAGE NO.
ABSTRACT	iii
SECTION 1 INTRODUCTION AND LITERATURE REVIEW	
CHAPTER I Introduction	1
CHAPTER II Current State of Knowledge of the Microbiology of Fleece Wool	3
CHAPTER III A Review of the Application of Chemicals to the Fleeces of Live Sheep	22
CHAPTER IV A Review of the <u>in vitro</u> Methods of Testing Antibacterial Chemicals	42
SECTION 2 EXPERIMENTAL	
CHAPTER V Introduction to Experimental Studies	64
CHAPTER VI Broth Dilution Turbidimetric and Agar Subculture Method	77
CHAPTER VII Agar Diffusion Method	83
CHAPTER VIII Manometric Method	97
CHAPTER IX Gradient Plate Method	111
CHAPTER X Field Experiment	118
SECTION 3 DISCUSSION AND CONCLUSIONS	
CHAPTER XI The Efficacy of the Chemicals Tested as Bacteriostatic Agents for use in Sheep Dips	136
CHAPTER XII Comparison and Discussion of the Methods Used to Assess the Antibacterial Activity of Test Compounds	142
ACKNOWLEDGEMENTS	149
REFERENCES	150
APPENDICES	170

SECTION I

INTRODUCTION AND LITERATURE REVIEW

CHAPTER I

INTRODUCTION

The practice of applying chemicals to the fleeces of live sheep has been carried on for centuries. Although these procedures have been primarily concerned with the control of arthropod ectoparasites of sheep, there have been a number of problems associated with the application of chemicals to sheep, or with the skins and fleeces of sheep that have been caused by micro-organisms. Problems involving micro-organisms have become important in more recent years due to changes in insecticide technology and for economic reasons.

This investigation was instituted to assess the effectiveness of six chemical agents as bacteriostats for use in sheep dipping baths. Before the evaluation of the compounds was begun, it was considered necessary to review the literature concerning the microbiology of fleece wool, and the history of the application of chemicals to the fleeces of live sheep.

As several laboratory methods were to be used in the evaluation of the test compounds, a critical review of the in vitro methods that have been used to test antibacterial chemicals was carried out. The reviews of the literature on these subjects make up the first section of this thesis.

Five methods were used to evaluate the test compounds. These were a broth dilution turbidimetric and agar subculture method, an

agar diffusion method, a manometric technique, a gradient plate method, and a field trial. The second section of this investigation outlines these methods and reports the results obtained from them.

The third section of the study involves a discussion of the results reported in Section 2, and some observations concerning the methods used. Finally recommendations on the use of the test compounds as sheep dip bacteriostats, and a standard method for testing chemicals for use as agents to control sheep dip and fleece micro-organisms are made.

CHAPTER II

THE CURRENT STATE OF KNOWLEDGE OF THE MICROBIOLOGY OF FLEECE WOOL

The microbiology of fleece wool is largely an ecological study involving considerations of the fleece as a habitat for fluctuating mixed populations of micro-organisms. It is therefore necessary to consider in particular the environment provided by the fleece of the live sheep as it may affect the growth of micro-organisms before considering the actual microbial populations that occur there.

According to Mulcock (1961), the reason the microbiology of fleece wool has been little studied is due to the hypothesis that micro-organisms are present in the fleece of sheep only as temporary contaminants and are therefore relatively unimportant. This supposition has been shown to be incorrect in recent years, due to the association of microbial populations with economically important and aesthetically undesirable conditions occurring on the skin and in the fleece of sheep.

In outlining the current state of knowledge of fleece microbiology, the fleece habitat will be considered first, followed by a summary of information concerning the microbial

populations that have been found to cause undesirable conditions in the fleece of the live sheep. Finally the microbial degradation of wool fibres will be discussed. This last section is considered separately because much of the study of this subject has been carried out from the point of view of the ability of micro-organisms to utilize wool fibres, rather than that of consideration of practical problems associated with the wool and associated industries.

I. THE FLEECE AS A HABITAT FOR MICROBIAL GROWTH

The fleece of the sheep is a composite of wool fibres and natural, acquired and applied impurities (Dusenbury, 1963). The fleece provides a habitat in which micro-organisms can successfully grow and multiply. Mulcock (1961 and 1965 b) considered the factors affecting the rate and amount of microbial growth in the fleece. These are reviewed under the headings of nutritional factors, temperature, hydrogen ion concentration and oxidation potential, radiations and water.

(1) Nutritional Factors

Adequate organic and inorganic nutrients are present within the fleece. Wool fibres consist largely of the protein keratin (Dusenbury, 1963). Natural impurities, including wool wax, suint and desquamated epithelial cells, contain many organic nutrients. Wool wax, the secretion of the skin sebaceous glands, contains,

according to Truter (1956), esters of water soluble alcohols and higher fatty acids as well as sterols, isocholesterols and some unidentified hydrocarbons. Suint, the dried secretions of the sudoriferous skin glands, contains a mixture of potassium soaps of fatty acids and minor amounts of lactic, hippuric and succinic acids, urea and lanaurin (Dusenbury, 1963). Acquired impurities, such as dust, sand, dirt, plant material and carbon from burned vegetation, are possible sources of nutrients for micro-organisms. Other impurities, including dusts and liquids applied to the fleece to improve the appearance of show sheep, or to control fleece-inhabiting insects, may also provide nutrients suitable for microbial utilization. The natural impurities however, are considered (Mulcock, 1961) to be the materials most generally available to micro-organisms because they are omnipresent in fleece wool.

(2) Temperature

The temperature conditions of the fleece are at least reasonable for microbial growth. The fleece of the live sheep reaches temperatures approaching the body heat of the animal (approximately 37°C) close to the skin surface. Also at this position temperature fluctuations are slight due in part to the relatively constant body temperature of the sheep and in part to the good insulation qualities of wool fibres. Temperature conditions at the distal end of the fleece fibres are far more variable, with temperatures being dependent upon that of the surrounding air.

Although fluctuations of temperature from below freezing in frosts to about 50°C in direct sunlight may be experienced, even so, several types of micro-organisms, as will be later described, can survive and even grow at this position. Fleece temperatures therefore, are usually not limiting for microbial growth.

(3) Hydrogen Ion Concentration and Oxidation Potential

Marks (pers. comm. 1972) measured the pH of a wide variety of wool types. She obtained a range of pH values of from 5.35 to 10.50. The pH optima for growth of most micro-organisms fall within this range. More acidic conditions may occur at the fibre tips than at other positions in the staple, due to the products of fibre photo-degradation (Speakman and McMahon, 1938), but it has been shown (Mulcock, 1965a) that microbial growth is possible even at this position on the staple.

The structure of the fleece is such as to assure a high state of oxidation, even when the fleece is saturated with water (Mulcock, 1961).

(4) Radiation

Many species of micro-organisms are susceptible to radiation. Light, especially the ultra-violet region of the spectrum, is well known to be injurious to many micro-organisms.

Levels of ultra-violet radiation vary quite widely over the fleece surface depending upon the degree of direct exposure to sunlight. Nevertheless, some micro-organisms can survive at the fibre tips (Mulcock, 1965a). Further down the fleece staple radiation levels are very low and microbial growth is not hindered by this factor in this region of the fleece.

(5) Water

The water content of the fleece is the most important factor determining microbial growth. Normally fleece water content is low, but after heavy rain, dipping, or to a lesser extent heavy dew, moisture levels are high (Fraser, 1957). It appears that in certain fleeces these levels are retained for long enough periods to support growth of microbial populations (Fraser, 1957). Micro-organisms that penetrate the skin surface can gain adequate water from the skin blood supply.

Thus, the fleece of the live sheep provides a habitat that can be successfully utilized by micro-organisms. It would appear that the base of the staple is the most suitable for microbial growth because of proximity of this area to fresh nutrients originating from the skin glands, and the lack of injurious levels of radiation. However, even the distal portion of the staple, as will be later indicated, is occupied by micro-organisms.

The results of Mulcock (1966) suggest that microbial populations are capable of survival for long periods in fleece wool. It therefore appears necessary to regard populations of micro-organisms in the fleece of the live sheep as a natural micro-ecosystem.

II. MICROBIAL DISORDERS OF THE FLEECE OF LIVE SHEEP

Many conditions of the skin and fleece of sheep which are directly or indirectly caused by microbial populations and which are, or may be, undesirable, have been observed. These disorders will be considered as those occurring at or near the skin surface, and those occurring at the distal end of the fleece staple. This division is necessary because these two areas are subject to different environmental conditions.

(1) Disorders Occurring At or Near the Skin Surface

The microbial disorders that occur at the skin surface or at the proximal end of the fleece staple of live sheep have been considered by Mulcock (1965b). In this review these conditions will be referred to under the following headings: (a) pyocyanine stains; (b) blue discolouration; (c) violet discolouration; (d) yellow discolourations; (e) red banding; (f) black banding; (g) pink rot; (h) fleece rot; and (i) ovine cutaneous actinomycosis.

(a) Pyocyanine Stains:

According to Seddon (1937), a green discolouration of wool, associated with an unidentified chromogenic micro-organism had been observed in 1894 by Professor Stuart of Sydney University. In 1929, again according to Seddon (1937), Seddon and McGrath easily isolated and cultivated Pseudomonas aeruginosa (Schroeter, 1872) Migula 1900, from green stained wool. Seddon (1937) reported the extraction of the pigment pyocyanine from stained wool and from cultures of Ps. aeruginosa. Mulcock (1961) extensively reviewed the literature concerning pyocyanine, a pigment produced exclusively by Ps. aeruginosa. Mulcock (1965b) noted that discolourations produced by Ps. aeruginosa in wool are diverse and often very striking. The colours found, as reported by Fraser and Mulcock (1956), were dependent upon the pH of the wool in which the organism is growing. Clean scoured wool stained with pure pyocyanine stained blue at pH 7.4 and above, blue-green at pH 5.8, green at pH 5.0, yellowish-brown at pH 3.0 and red at pH 2.0. These authors suggested that the red colour that occurred towards the distal end of naturally stained staples could be due to the acid products of fibre photo-degradation (Speakman and McMahon, 1938).

It would thus appear that the pigment pyocyanine produced by Ps. aeruginosa could be responsible for blue, green, yellowish-brown or red discolourations of fleece wool.

(b) Blue Discolouration

A blue discolouration of the fleece, attributed to a pigment produced by Chromobacterium coeruleum (Smith) Ford 1927, was described by Seddon (1937). Mulcock et al (1965) described a similar condition in fleeces collected from the South Island of New Zealand. The pigment in the wool was shown by these authors to be produced by the organism Pseudomonas indigofera Elazari-Volcani, 1939. The pigment, which was in the form of small particles, could be easily washed from stained fleeces.

(c) Violet Discolouration

Seddon (1937) described a violet discolouration in wool and attributed it to Chromobacterium violaceum (Schroeter, 1872) Bergonzini, 1881, although his evidence for this was not entirely satisfactory. Mulcock (1965b) noted that the condition was quite frequently found in New Zealand fleece wool.

(d) Yellow Discolourations:

Several reports of yellow discolourations in wool have been made and these are reviewed by Mulcock (1961) and Hoare (1968). Although Hoare (1968) suggested that micro-organisms could possess enzyme systems capable of inducing wool yellowing by an oxidative mechanism, as yet these colourations have not been definitely associated with micro-organisms. Mulcock (1961) postulated that golden yellow fleece

colourations may become very much deeper yellow due to microbial growth when affected wool is packed in bales.

(e) Red Banding:

A red banding condition in fleece wool was recorded by Seddon (1937), and it was suggested that this may be associated with populations of Serratia marcescens (Bizio, 1823). Mulcock (1965b) pointed out that the one sample with red banding examined by him did not contain any chromogenic micro-organisms. He pointed out that the condition was rare.

(f) Black Banding:

Catan and di Rocco (1965) noted a condition of fleece wool in which blackish or dark grey zonal bands associated with an agglutinated appearance at the medium part of the fibre occurred. In some samples a skin dermatitis was also present. Streptomyces niger (Krainsky, 1914) Waksman and Henrici 1948, was isolated from the blackened wool. Fibres attacked by this organism showed alterations in morphological structure. In vitro observations showed that a similar condition occurred.

(g) Pink Rot:

Waters (1932) described a pink-rot condition of fleece wool, and isolated an organism from affected wool which closely resembled Bacillus vulgatus (Bacillus subtilis Cohn, 1872). He made the tentative suggestion that the organism could be a variety of the species. The condition was characterized by a pink colouration of varying intensity and by the matting, clogging

and weakness or breakage of the wool fibres. Molyneux (1959 and 1961) reported the isolation, characteristics and proteolytic properties of an aerobic, mesophilic, spore-forming bacillus considered to be a variant with characteristics between those of Bacillus subtilis and B. pumilis, from the contents of an experimentally induced dermoid cyst implanted on the mid-lateral line of a single sheep. He drew a comparison between the intracystic environment of the wool and the environment necessary for the development of pink-rot.

(h) Fleece Rot:

Fleece rot was first recognised and described by Seddon (1931) and was defined by Belschner (1937) as a condition due to the multiplication of bacteria on the skin and in over-lying wool. Belschner (1937) ascribed the condition to high fleece moisture contents leading to a partial maceration of the surface of the skin allowing bacterial multiplication to occur in the damaged skin cells. A serous exudate resulted from this superficial dermatitis and caused, after drying, a band of crusty matted fibres across the staple which was raised from the skin as the wool continued to grow. Also discussed by Belschner (1937) were differences in susceptibility between sheep to fleece rot. It appeared that young sheep, those with open fleeces particularly on the withers, and with yellow, harsh and poor wool characters were more susceptible to the condition.

Various workers, including Hayman (1953) and Jeffries (1961) have further studied this conditions, but apart from minor modifications to Belschner's (1937) original characterizations, have added little to the knowledge about this disorder. No particular organism has been associated with the condition. It would appear however, that the organisms responsible are part of the normal fleece microbial population. As Belschner (1937) noted, "The organisms for it (fleece rot) are probably present in the fleece as a result of contamination by dust, etc."

(i) Ovine Cutaneous Actinomycosis:

Roberts and Graham (1966) noted that ovine cutaneous actinomycosis (mycotic dermatitis or 'lumpy wool') is characterized by the continuous production over a period of months or years of scab material that is bound by the wool fibres into compact horny masses. In this condition the wool fibres are not typically damaged. However, a certain amount of break may be associated with the disease due to a check in general condition of the animal following a severe attack of the disease (Henderson, 1955), or to secondary bacterial attack on the wool fibres associated with the scab material, especially when it is wet (Lipson quoted by Roberts and Graham, 1966). It appears that rainfall is important in initiating the disease (Pulsford, 1967), although once the disease is established the dry impervious scab prevents dessication of the skin lesion (Roberts, 1957b).

The microbiological aspects of ovine cutaneous actinomycosis

have been quite widely studied. There has been some controversy over the classification of the actinomycete involved. The organism was first isolated by Bull (1929) and named Actinomyces dermatonomus. Previously van Saceghem in 1915 (cited by Roberts, 1961) described a disease of cattle in the Belgian Congo, and named the associated organism Dermatophilus congolensis. Thompson and Bissett (1957) described the isolation and characterization of the causative organism of strawberry footrot of sheep and named it Polysepta pedis. Austwick (1958) showed that the genus Polysepta of Thompson and Bissett was synonymous with the genus Dermatophilus, with the latter being given priority. However, Gordon (1964), after extensively studying the characteristics of seventeen Dermatophilus strains from skin lesions of cattle, sheep, horses, deer and man, concluded that all isolates could be accommodated in the species D. congolensis Van Saceghem, 1915, emend. 1916, 1934, with D. dermatonomus and D. pedis falling into synonymy.

The causative organism has been extensively studied, particularly by Roberts (1957b and c; 1961; 1953a, b, c, and d; and 1964). The life cycle of the organism was outlined by Roberts (1961 and 1962). Infective zoospores are released from the lesion, and these, after swimming in moisture layers on wet wool and skin, settle on a susceptible skin area. There they germinate, and produce a small germ tube, which passes into the skin and extends as a branching "hypha" into the skin tissues, causing skin damage and the characteristic inflammatory response.

As the surface "hyphae" age, they undergo divisions leading to the formation of many new zoospores. These escape to the exterior and infect any other susceptible areas on the same sheep, or may be transferred either by direct contact or by insect vectors, especially flies (Richard and Pier, 1966) to other sheep. It appears that the very common minor infections of the disease on the faces and ears of sheep act as chief sources of infection, and that severe outbreaks covering the whole animal may be due to wet conditions or other circumstances resulting in prolonged wetting of the fleece.

(2) Disorders Occurring at the Distal End
of the Staple.

The second division of conditions of the fleece caused by micro-organisms made by Mulcock (1965b) is that in which he considered those occurring at the fibre tips. It appears that three main disorders are known.

(a) Red-tip.

The occurrence of a pink or red discolouration at the tips of wool fibres of fleeces was recorded briefly by Seddon (1937). Henderson (1955) considered the problem in more detail, and noted that the condition is invariably associated with a high degree of weathering of the fibre tips. Henderson further noted that examination of pink tipped wool showed the presence of many chromogenic organisms including un-named micrococci and

some yeasts. The condition occurred in 0.51% of over 42,000 inspected fleeces of Romney and crossbred sheep, the group in which it was most prevalent. Because of this low incidence, Henderson considered that the disorder "cannot be regarded as a serious fault".

(b) Fungus Tip.

The condition known as "fungus tip" was first noted as a disorder of fleece wool by Mulcock (1959), who later published more extensive studies on the subject (Mulcock, 1961 and 1965a). A black, non-scourable discolouration of the fibre tips was noted and associated with the hyphae and fruiting bodies of a fungus classified as Peyronellaea glomerata (Corda) Goidanick. Mulcock (1965a) pointed out that this fungus occupies a unique habitat in that it can thrive on the distal ends of the fibres exposed to extremes of temperature and solar radiation, the ultra-violet portion of which may be damaging to micro-organisms. The uniqueness of this organism is questioned, however, when it is noted that other micro-organisms have been recorded at this position on the fleece staple (Henderson, 1955).

(c) Fungal Discolourations

Jacks (1959a and b; 1960) reported the isolation of several pigment producing fungi from a sample of wool stained grey and yellow to reddish brown. The stains were unscourable. He attributed the presence of these organisms to contamination

of the fleece wool from fungal populations occurring on plants and in the soil. It seems reasonable that the stains could have been caused by the Phoma, Aspergillus, Chaetomium, Epicoccum, Stemphylium and Macrosporium spp. living on acquired impurities in the fleece, and isolated by Jacks (1959a and b). However, as Mulcock (1965b) pointed out, further investigation of the problem is needed before some of the assertions of Jacks can be accepted.

III. THE MICROBIAL BREAKDOWN OF WOOL FIBRES

Several workers have shown the ability of micro-organisms to attack wool and other fleece substances. However, all studies have been in vitro, and the importance of the findings in the microbiology of the fleece of live sheep can only be speculated upon.

Philipson (1957) found that un-named Actinomycetes isolated from raw wool were capable of decomposing both unsterilised and autoclaved wool under humid conditions at 37°C. She also found that Actinomycetes isolated from soil could degrade wool under similar conditions.

Noval and Nickerson (1959) showed that a strain of Streptomyces fradiae (Waksman and Curtis, 1916) Waksman and Henrici, 1948, isolated from soil possessed the ability to digest keratin of native wool and chicken feathers rapidly and completely. These authors suggested that the mechanism of

digestion was a combination of reducing and proteolytic agents, neither of which alone could account for the extent of keratin decomposition accomplished. Further, they suggested that strains of S. fradiae were unique in their ability to digest native keratin; 80 to 90% of the dry weight of wool and feathers were rendered soluble under optimum conditions, which were agitation at 37°C in a medium with initial pH 7.7, containing keratin as the sole source of carbon and nitrogen.

Molyneux (1961) considered the mechanism of fibre degradation of the organism he had previously isolated (Molyneux, 1959). He concluded that the degradation was characterised by two mechanisms; the reduction of the -S-S- cross linkages of the keratin molecule, and proteolysis respectively. This degradation proceeds to the disintegration of the microfibrils of the wool.

McQuade (1964) tested approximately one hundred species of bacteria, streptomycetes, and fungi for their in vitro ability to decompose wool. Wool, which had been sterilized by processes designed to prevent protein denaturation, was decomposed by widely different micro-organisms. It was suggested that the ability to decompose keratin was necessary, but not the only condition, for pathogenicity (sic) of wool fibres and that alkalinity was necessary for the microbiological degradation of keratins. McQuade (1964) discussed difficulties of interpreting data obtained from in vitro studies in relation to natural saprophytism and parasitism

(sic) of keratinous tissues.

Shaposhnikov et al., (1964) noted that a great number of micro-organisms on the surface of wool could utilize wool fibres as a substrate for their development, and thus cause fibre disintegration. The most active organisms noted were Actinomyces globisporus, (Krassilnikov, 1949) and Bacillus mesentericus, with B. cereus (Frankland & Frankland, 1887) and B. sublustris also active. Paecilomyces varioti, a mould, was a quite active fibre decomposer while Aspergillus and Penicillium spp. grew only at the expense of "wool fat". These workers also noted that the development of microbes in wool fibres was accompanied by the appearance in the culture fluid of free amino acids and ammonia, a fact formerly noted by Noval and Nickerson (1959). The mechanism of decomposition suggested by the more recent work was that the microbes caused ammonification of the keratin, resulting in fibre disintegration.

Feniksova and Petrova (1965) found that when a strain of Actinomyces fradiae was grown in a mineral medium with sheep wool as the sole source of carbon and nitrogen, enzymes which dissolved wool accumulated in the culture liquid. Cell free culture liquid dissolved up to 2.5 to 3.0% of wool. It was suggested that wool induced synthesis of the proteolytic enzymes by Actinomyces spp., or was the source of a compound necessary to the organism, but missing in the synthetic growth medium used as a comparison with raw wool.

IV. CONCLUSIONS

The microbial ecology of fleece wool involves populations of parasitic, facultatively saprophytic and saprophytic micro-organisms living in the habitat provided by the live sheep, wool fibres and associated fleece impurities, and affected by external environmental conditions. That the growth of such populations causes undesirable conditions in wool is well established. That these conditions are not normal is due only to the fact that the environmental conditions required for their expression are comparatively uncommon, not that the micro-organisms themselves are uncommon.

There are areas where knowledge of wool microbiology is lacking. It is quite widely appreciated that the occurrence of large numbers of specific micro-organisms in the fleeces of sheep is dependent upon factors peculiar to individual animals. For example, Hart (1967) found that the incidence of ovine cutaneous actinomycosis varied widely within and between sheep flocks. He recorded a range of infection of from 13 to 95%. Fraser (1957) showed that fleeces apparently more susceptible to fleece rot dried out more slowly after wetting than those less susceptible. Clearly variations in fleece structure are involved, but the effects of variations in pH, nutrient and other chemicals, temperature, aeration, and radiation, although obviously important are not fully understood. It is in the field of fleece microbial ecology that further investigation

would be fruitful.

Studies on the microbiology of fleece wool have been both scientifically and practically significant. The need for higher quality in natural fibres due, at least in part, to the increased competition from synthetic fibres in the fibre industries (Hoare, 1968), has made it necessary for many of the problems posed by microbial disorders of fleece wool to be solved. In the solution of these, valuable microbiological knowledge has also been gained.

CHAPTER III

A REVIEW OF THE APPLICATION OF CHEMICALS
TO THE FLEECES OF LIVE SHEEP

From the literature review in the previous chapter, it is clear that the fleece of the living sheep provides a habitat for micro-organisms, some of which may be undesirable. Arthropod skin parasites have also been known to cause sheep health problems. These organisms can cause serious economic loss by damage to wool and pelts, and by reducing the condition of infested animals so that growth rates are lowered and wool production decreased.

The control of micro-organisms and arthropods in the fleeces of sheep has almost invariably been attempted by the application of chemicals to the fleece. The methods of application of these materials to sheep have been reviewed by Endrejat (1967). He pointed out that up until the 1940s local treatments, such as ointments, were often used. In the treatment of large numbers of sheep animals were dipped in solutions or emulsions of effective materials, originally in small tubs and later in large dipping baths. Swim baths, or "plunge dips", of long or circular shape containing several hundred gallons of liquid have been used to totally immerse the animals and thus completely cover the fleece with the effective materials.

More modern methods, including high volume showers and sprays, which completely saturate the fleece, and low volume tip sprays and dust sprays, which apply chemical to the fibre tips from where it can diffuse down the fibres to the skin surface, have been developed. Local treatment of skin areas particularly susceptible to arthropod attack may be accomplished using a high pressure pump and a hand spray gun, the procedure being referred to as "jetting".

In New Zealand, under the Animals Act, 1967 (New Zealand Statutes, 1967), it is compulsory for all farmers to apply chemicals to their sheep to control the arthropod parasites, lice and keds. An approved method of chemical application must be used and a suitable chemical must be applied within a specified period after the sheep have been shorn. In this country the application of chemicals to the fleeces of sheep is thus part of the yearly husbandry programme of every sheep farmer.

This review considers the application of chemicals to the fleeces of live sheep under the following headings:

- (1) arthropod ectoparasites of sheep and their control
- (2) control of undesirable micro-organisms in the fleece
- (3) microbial problems associated with sheep dipping and their control

I. ARTHROPOD ECTOPARASITES OF SHEEP AND THEIR CONTROL

The history of the application of chemicals to the fleece of live sheep closely parallels the history of the problems caused by arthropod ectoparasites of sheep. For this reason, although the arthropods concerned are here considered separately from chemical control measures, both are closely inter-related.

(1) Arthropod Ectoparasites of Sheep

The important arthropod ectoparasites of sheep are:

- (a) the psoroptic mange mite
- (b) the sheep ked
- (c) sheep lice
- (d) flies
- (e) scrotal mange mite and itch mite

These will be considered separately.

(a) The Psoroptic Mange Mite:

The psoroptic mange mite, Psoroptes ovis (Hering, 1838, Gervais 1941), and the condition it causes, sheep scab, have been known since antiquity (Endrejat, 1967). Scabbed sheep were among the animals forbidden to be "offered by fire ... upon the altar of the Lord" by Moses in the book of Leviticus, Chapter 22, verses 21 and 22, which was written about 1490 B.C.

Psoroptic mange mites puncture the epidermis of the sheep and suck lymph from the skin tissues. The puncturing of the epidermis causes a local reaction in the form of a small inflammatory swelling richly infiltrated with serum. The serum exudes onto the skin surface and coagulates, thus forming a crust (Soulsby, 1968). It is from

this crust that the clinical signs and the common name of the condition are derived. Jubb and Kennedy (1970) noted that the lesions itch so severely that sheep rub, scratch and bite the wool, and the fleece becomes irregular, matted and may be shed.

Psoroptic mange mite has been of major importance in several areas of the world. After its introduction into Australia in 1788, sheep scab became a major problem. Orders were made restricting the movement of infested sheep, and requiring the slaughter of flocks harbouring the parasite, in 1831 and 1854 respectively, but these attempts at eradication were unsuccessful (Endrejat, 1967). In 1864, double dipping, in which sheep were dipped twice over a period of about two weeks, in suitable miticide materials, was introduced in Australia, and this measure enabled the complete eradication of sheep scab by 1896 (Seddon, 1964).

Psoroptes ovis was eradicated from New Zealand at about the same time, after methods similar to those used in Australia had been adopted. The parasite has been eradicated more recently from Great Britain by 1952, and from South Africa, Uruguay and West Germany by 1962. The disease still remains a problem of economic importance in some parts of Eastern Europe, Asia, Africa and America (Endrejat, 1967).

(b) The Sheep Ked:

The sheep ked, Melophagus ovinus (Linné, 1758) is a wingless fly of the family Hippoboscidae, and is an obligate parasite of sheep in all stages of its development (Jubb and Kennedy, 1970). Soulsby (1968) noted that these parasites live in the wool of the sheep and suck blood from the skin tissues, producing intense irritation and causing the sheep to bite, rub and scratch themselves, bringing about fleece damage. Heavy infestations can reduce the condition of the host considerably, and may even cause anaemia (Soulsby, 1968). The same author also reported that keds may transmit the widespread non-pathogenic (sic) protozoan Trypanosoma melophagium. As well, the faeces of keds can cause wool stains that are only partially scourable (Henderson, 1968).

Sheep keds are serious parasites in almost all countries where sheep rearing is important. These insects have survived despite the control measures against them.

(c) Sheep Lice:

Two groups of lice attack sheep. These are the head, or blue lice, and the leg lice.

The head or blue louse, Linognathus ovillus (Neumann, 1907), sometimes referred to incorrectly as the body louse, is a sucking insect which inhabits the hairy areas of the fleece other than the legs. This insect is of little importance in New Zealand, but in countries where it is common the severe irritation and discomfort it causes to infected animals makes control measures necessary.

Two species of lice inhabit the skin of the legs of sheep. They are the sucking louse Linognathus pedalis (Osborn, 1896) and the biting louse Damalinia ovis (Linné, 1758) (Murray, 1963). Severe attacks may result in lameness of sheep, Jubb and Kennedy (1970) noted that D. ovis can also attack wool-covered areas of the skin causing considerable irritation. The result is that infested sheep rub against fences, trees and other objects, which gives rise to a bedraggled appearance, with some loss of wool.

Sheep lice, like sheep keds, have survived large scale control measures in almost all countries where sheep production is important.

(d) Flies:

Flies are important ecto-parasites of sheep in Australia, New Zealand, South Africa, and Great Britain where cutaneous myiasis, or blowfly strike can cause important economic loss (Endrejat, 1967). Two types of flies attacking sheep have been recognized (Belschner, 1965). These are primary and secondary flies.

Primary flies are attracted to moist areas of the fleece and skin that may be contaminated with urine and faeces and where bacterial activity is high. The area most commonly infested is the hindquarters, but all portions of the body may become struck. Secondary flies, are those that are attracted to areas of skin where primary flies are active.

Primary flies lay eggs on the wool. These hatch, and the larvae, or maggots, move down the wool fibres and begin feeding on the skin tissues. An acute dermatitis is produced in which there is considerable exudation from the skin and bacterial activity (Jubb and Kennedy, 1970). The lesions thus become attractive to secondary

flies, the larvae of which feed similarly to those of primary flies.

The important species of primary flies include Lucilia cuprina Robineau-Desvoidy, Calliphora augur Hardy, Calliphora stygia Fabricus, and Lucilia sericata Robineau-Desvoidy. The more important secondary flies are Chrysomya rufifacies Macquart, Chrysomya pogon Bigot, Microcalliphora varipes and Sarcophaga Meigen sp. (Belschner, 1965).

The control of fly strike in sheep is usually achieved by local applications of chemicals, using jetting techniques, to susceptible areas of the fleece. This is done at times when flystrike is most likely to occur, that is, in warm humid weather. Eradication of blowflies is almost impossible because these insects commonly live on carrion, the parasitic habit being a secondary one (Jubb and Kennedy, 1970).

(e) Other Sheep Ectoparasites:

Two members of the class Arachnida, namely Psorergates ovis Wornersley, and Chorioptes bovis (Hering) Gervais and van Beueden, the itch mite and scrotal mange mite respectively are of minor importance as sheep ectoparasites. The itch mite may cause a mild, but chronic irritation in affected sheep (Soulsby, 1968) while the scrotal mange mite, which may attack the skin of the scrotum of rams, (Jubb and Kennedy, 1970), can have adverse effects on ram fertility.

Itch mite is quite easily controlled and causes only slight problems where it occurs (Endrejat, 1967) and scrotal mange mite can be controlled using local applications of chemicals to the scrotum of affected rams.

(2) Chemical Control of Sheep Arthropod

Ectoparasites:

The control of arthropod parasites of the sheep's skin using chemicals has been carried out since antiquity. The materials used fit into a clear chronological sequence.

The earliest recorded problem involving sheep ectoparasites was that of sheep scab, and it was to control this condition that the use of chemicals was first reported. Vergil's "Georgics", which was written between 37 and 30 B.C., contains references to the use of sulphur, pitch and wax to control loathsome scabs affecting sheep (Endrejat, 1967). "Cato's ointment", named for a senator of the Roman Republic who lived between 234 and 149 B.C., consisted of a mixture of water and the dregs of olive oil, boiled with lupins and the lees of wine. This was rubbed into scabbed areas of the skin of affected sheep, and after a period of two days, was washed off with salt water (Endrejat, 1967).

It was not until the middle of the 19th century however, that significant advances were made in the chemical control of sheep scab. In New Zealand, according to Laing (1964), a nicotine-sulphur dipping solution was recommended in 1854 for the control of scab. Lime-sulphur dips became popular for the same purpose in the 1860s in New Zealand (Laing, 1964). The use of nicotine-sulphur and lime-sulphur dips in New Zealand probably stemmed from their use to good effect in Australia, where sheep scab was eradicated by 1896.

At about this time, the first attempts to control sheep scab with arsenical materials were being made. In 1810 the Frenchman Tessier dipped sheep in a solution of arsenic, but wool staining and death by poisoning of some of the sheep resulted (Cooper, 1960). Between

1843 and 1852 William Cooper, an Englishman, developed an arsenic-sulphur dipping material, and began producing it on a commercial basis (Cooper, 1960). This dip was introduced into New Zealand in the 1870s, and aided in the eradication of sheep scab (Laing, 1964).

In 1852, the Scotsman Alexander McDougall patented a so-called non-poisonous dip based on creosote and carbolic acid (Cooper, 1960). Cresylic acid dips were used in New Zealand during the first part of this century, mainly for dipping young sheep (McLean, pers. com. 1971).

In 1923, derris root, containing the active ingredient rotenone, was incorporated with an arsenical dipping material to improve efficiency of control of keds, and to speed up lice kill (Cook, pers. com. 1971). This combination is still in use in some parts of Australia.

The use of arsenic and cresylic acid based dipping materials became unpopular with farmers because poisoning and so-called "arsenical scald" occurred. Scalding appears to be caused by the alkaline conditions present in arsenical dip solutions (Graham, pers. com. 1971) and is characterised by reddening and tenderness of the skin of affected animals. In more severe cases a dermatitis, characterised by intense erythema, necrosis and sloughing of the skin and wool (Jubb and Kennedy, 1970) may occur.

Various chemicals have been tested as possible alternatives to arsenic and cresylic acid preparations. Morrison and McLeod (1941) suggested the use of derris powder in suspension without other chemicals. These workers showed that derris suspensions were particularly effective for the control of keds.

Toward the end and immediately after World War II, the chlorinated hydrocarbon insecticides became commercially available. Coop and McLeod (1949) showed in New Zealand that the gamma isomer of benzene hexachloride (gamma-BHC) also known as lindane, was an effective agent against both lice and keds.

Following the successful use of gamma-BHC, other chlorinated hydro-carbon insecticides, showing even greater insect control capabilities were used as sheep dipping chemicals. These included aldrin, dieldrin, endrin and heptochlor, which at the time appeared to be ideal insecticides. Only small amounts were needed in the fleece to give complete and long term control of the important ectoparasites. Thomas (1958) compared arsenic, derris, gamma-BHC, aldrin and dieldrin for their efficiency in controlling lice and keds. He found that arsenic and gamma-BHC were not as effective as were aldrin and dieldrin.

The high level of efficiency of the chlorinated hydrocarbon insecticides in controlling the major ectoparasites of sheep made total immersion of sheep in insecticide preparations unnecessary. Plunge dipping procedures became obsolete, and were replaced by spraying, tip spraying and dusting procedures, which were more economical, as they saved both time and labour. Thus, with a change in insecticide chemicals came a change in dipping methods.

The chlorinated hydrocarbon insecticides became widely used in agriculture for both crop protection and animal ectoparasite control (Endrejat, 1967). Residues of these chemicals were detected in the body fat of animals eating plant material previously treated with them, in animals to which they had been directly applied, and in humans eating contaminated animal products (Collet and Harrison, 1963;

Le Breton, 1963). The possibility that residues in food for human consumption could be harmful, directly or indirectly caused many governments, including that of New Zealand, to ban the use of chlorinated hydro-carbon insecticides in the early 1960s (Endrejat, 1967).

Again alternative materials were required for the control of sheep ectoparasites. Organophosphorus compounds had been shown to control lice and keds (Thomas, 1958) and these compounds have now become almost universally adopted for this purpose. Heath and Millar (1970), in New Zealand, tested sixteen insecticides comprising ten organophosphorus compounds, five carbamates and one inorganic compound against Damalinia ovis and Melophagus ovinus. The majority of the compounds tested gave reasonable control of both organisms, but diazinon (0,0-diethyl 0-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate) and 3,5-di-tertiary butylphenol N-methyl carbamate were most effective. Diazinon is now the most widely used insecticide for the control of sheep ectoparasites.

Organophosphorus compounds are not as effective as were the chlorinated hydrocarbons. In order to kill the parasites, complete immersion of the host animal is necessary. It is now recognized that plunge dipping is the best method of controlling lice and keds with the chemicals available (Graham, pers. comm. 1971). Plunge dipping is thus becoming a widespread procedure once more.

II. THE CONTROL OF UNDESIRABLE MICRO-ORGANISMS OF THE FLEECE

The undesirable micro-organisms of the fleece of the live sheep have been discussed in the previous chapter. Of these, only Dermatophilus congolensis has been successfully controlled using applications of chemicals to the fleece. The materials that have been suggested for the control of ovine cutaneous actinomycosis are now reviewed.

In 1929, Bull (1929) tested iodine, copper sulphate, mercuric chloride, and sulphuric acid in vitro against D. congolensis. All were effective at high concentrations while mercuric chloride was most effective at low concentrations. Copper sulphate was recommended, however, because it was easy to acquire, cheap and of comparatively low toxicity.

Zlotnik (1955) cited work of Malfroy, who in 1938 claimed that a solution containing 3g. picric acid and 3g. copper sulphate in 1,000g. water gave beneficial results in curing the early stages of cases of Senkobo disease of cattle, caused by D. congolensis. Zlotnik (1955) further cited the work of Curasson and Mornet, who in 1941 noted that arsenical dips had a definite curative effect on the same disease. Zlotnik (1955) himself reported the results of trials with a quaternary ammonium compound applied to infected areas of the skin of cattle. The compound gave excellent results with recent cases, which were cured within two weeks.

Roberts (1957c) reported that copper and zinc sulphates were more effective in the control of hyphal growth of D. congolensis than of coccoid cell motility, implying that these compounds could be of

use as curative rather than preventative remedies.

Roberts and Graham (1966) and Roberts (1967) suggested that spraying sheep with a 0.5% solution of zinc sulphate within two hours of shearing should prevent infection of shearing injuries by D. congolensis.

Hart et al., (1967) carried out agar cup diffusion tests, tests in fluid media, and bactericidal tests of a large number of organic and inorganic compounds. Several showed bacteriostatic and bactericidal effects against D. congolensis.

Hart (1965) and Hart and Tyszkiewicz (1968) reported that the application of 1% potassium aluminium sulphate (alum) by dipping was beneficial against D. congolensis infections of sheep, and that this effect was more pronounced and prolonged than those of 0.2% copper sulphate, 0.1% 2:4:5-trichlorophenol, and 0.128% butyl 2:4-dichlorophenoxyacetate. Dipping in 1% alum gave significant improvement of natural infections. Backwashing with 1% alum, or dusting with 57% anhydrous alum in an inert carrier gave highly and very highly significant improvement respectively over controls.

The control of other undesirable fleece micro-organisms using chemicals has not been attempted. Pyocyanine stains, blue, violet and yellow discolourations, red and black banding, pink rot and fleece rot, all of which are attributable to micro-organisms, are conditions of the fleece that reduce the economic value of affected wool. For this reason the control of these conditions would be desirable. One of the purposes of this study is to assess the practicability of the control of some of the micro-organisms that cause undesirable conditions in the sheep fleece.

III THE MICROBIAL PROBLEMS ASSOCIATED WITH SHEEP DIPPING AND THEIR CONTROL

(1) The Microbial Problems Associated with Sheep

Dipping:

Several problems involving micro-organisms have been encountered where sheep are dipped in plunge dips. Plunge dipping which was widely used before the chlorinated hydrocarbon insecticides became available, seems likely to again become popular because it is now realized that plunge dipping is the most efficient method of application of materials to the fleeces of live sheep (Graham, pers. com. 1971).

In plunge dipping procedures large numbers of sheep are dipped in the same dip wash, and often this wash is kept in the dipping bath for several days. This allows both the transmission of organisms from one sheep to another, and also microbial multiplication to take place by the utilization of the soluble fleece constituents, dust, excretory and faecal materials. Four problems involving micro-organisms have arisen from plunge dipping. These are:

- (i) post-dipping lameness
- (ii) dip wash blackening
- (iii) transmission of ovine cutaneous actinomycosis
- (iv) post-dipping pneumonia

(a) Post-dipping Lameness:

McLean (1948) published the first report of post-dipping lameness (PDL). He described the condition as a lameness

occurring after a constant incubation period, following the dipping of sheep in washes containing rotenone (derris root) and benzene hexachloride. The symptoms were associated with leg cuts and abrasions. He observed that sheep dipped in freshly prepared dip washes, or in washes containing bacteriostatic concentrations of tar acids, did not develop symptoms. The comparatively constant incubation period, and the lack of bacteriostatic action of the dipping material used suggested that the problem was a microbiological one.

Whitten et al., (1948) described an investigation into PDL. They found that the incidence of lameness increased with increasing fouling of the dip wash. The bacterium Erysipelothrix rhusiopathiae (Migula) Buchanan 1918 was isolated from affected animals and its pathogenicity proven. These workers suggested that bacteriostatic agents should be used in association with non-bacteriostatic insecticides used for sheep dipping.

Whitten (1952) reported an outbreak of lameness in sheep caused by E. rhusiopathiae, but not associated with dipping. In this case infection appeared to have come directly from soil.

It appears then, as outlined by Wright and Pelham (1968) that PDL is caused by E. rhusiopathiae infection when sheep with broken skin on the legs are dipped in fouled dip wash. The organism originates from soil, and is of low pathogenicity, but is capable of rapid multiplication in dirty insecticide solutions not containing bacteriostatic compounds. Thus, antibacterial agents should always be included in plunge dipping mixtures to prevent this condition.

(b) Dip Wash Blackening:

If dip wash free of bacteriostatic agents is left to stand in dip baths for several days, it may become blackened. The author has observed blackening occurring under laboratory conditions after four days in a wash that had been used to spray approximately 600 mature sheep. This wash contained the insecticide 2,4-dichlorophenyl diethyl phosphorothionate with no added bacteriostat, and was held in an open 2.5 litre container at 25°.

Dip wash blackening is caused by sulphur reducing strains of Escherichia coli (Migula 1895) Castellani and Chalmers, 1919, that enter the dip wash by faecal contamination (Graham, pers. com. 1971). Blackened dip washes are aesthetically undesirable and can cause wool discolourations (Graham, pers. com. 1971). The fact that some strains of E. coli may be pathogenic is of greater importance however. For example, Bruner and Gillespie (1966) cited work of Howarth, who in 1932 reported a disease in pregnant ewes resulting in abortions and a high mortality rate. E. coli was isolated from the foetal body fluids and organs and from the genital tracts of affected ewes.

Roberts (1957a) reported an outbreak of a disease in lambs, characterised by heavy mortality, in which diarrhoea and a secondary severe meningitis were observed. A hydrogen sulphide producing strain of E. coli was isolated as a pure culture from the cerebrospinal fluid, liver and small intestine of affected lambs.

Charles (1957) described an E. coli infection in lambs similar to that reported by Roberts (1957a), except that a severe meningoencephalitis was the primary symptom, with there being very little evidence of diarrhoea.

Roberts (1958) described a further outbreak of E. coli septicaemia in lambs in which arthritis and diarrhoea were outstanding features. E. coli was isolated in pure culture from liver, pericardial and joint biopsies of affected animals.

Although no outbreaks of E. coli infection have been associated with sheep dipping, the varied and often fatal diseases this organism can cause make it undesirable for large numbers to come into contact with healthy sheep. This, coupled with the blackened dip wash caused by the ability of some strains to reduce sulphur make their control important.

(c) Transmission of Ovine Cutaneous Actinomycosis

Roberts and Graham (1966) noted that if dipping was delayed longer than two weeks after shearing, the longer wool staple increased the probability of the occurrence of skin scalding, due to delayed drying of the fleece. They pointed out that dip scalded skin was very susceptible to infection by Dermatophilus congolensis.

Le Riche (1967) suggested that transmission of ovine cutaneous actinomycosis could occur via dipping fluids. Lesions of D. congolensis are very common on the faces and ears of sheep. It seems reasonable that transmission from these lesions to uninfected areas of skin on other parts of the bodies of both infected and uninfected sheep is possible through dipping fluids. The motile zoospore or "coccoid" stage of D. congolensis would presumably be responsible.

(d) Post-dipping Pneumonia

Post-dipping pneumonia has been responsible for heavy sheep losses following plunge dipping. The cause of this condition is not known. Various possible causes have been suggested (Graham, pers. com. 1971). These include the inhalation of arsenical dipping fluids, rough handling of sheep during dipping, cold weather conditions after dipping, or an infection by bacteria following attack by an infective agent, possibly a virus.

(2) The Control of the Microbial Problems Associated With Sheep Dipping

Prior to the mid-1940s the problems of post-dipping lameness, dip wash blackening, and ovine cutaneous actinomycosis transmission were not apparent. This was due to the fact that arsenic, a very effective bacteriostat, was widely used in dip washes as an insecticide. With the change to non-bacteriostatic insecticides, in the first place derris root and the chlorinated hydrocarbons and later organophosphorus compounds, dipping problems associated with micro-organisms became apparent (McLean, 1948). To control these problems bacteriostatic materials have been added to dip washes. Some of the materials that have been suggested for use as dip bacteriostats are now reviewed.

Whitten et al., (1948) suggested that the addition of copper sulphate to plunge dips would control post-dipping lameness. However, this material proved unsatisfactory because it caused wool discolouration.

Wright and Pelham (1968) suggested that tetramethylthiuram-disulphide would control Erysipelothrix rhusiopathiae in sheep dips.

They noted that this material had been successfully used in the field to control post-dipping lameness. Tetramethyl thiuram disulphide is now commonly used to control both E. rhusiopathiae and sulphur reducing bacteria (Graham, pers. com. 1971).

Arsenic is still used as a dip bacteriostat (Graham, pers. com. 1971), although arsenical scalding may give problems. A 1.25% solution of sodium arsenite, approximately half that needed to control itch mites, is recommended to prevent post-dipping lameness and dip wash blackening (Tasman Vaccine Laboratory Limited, New Zealand; label recommendations).

Graham (pers. com. 1971) observed that carbolic and phenolic compounds have been tested as sheep dip bacteriostats, but had little effect. He pointed out that magnesium fluosilicate is now frequently used to prevent post-dipping lameness and dip wash blackening.

Roberts and Graham (1966) suggested that if antibacterial agents were present in dip washes at rapidly bactericidal concentrations throughout dipping, they should prevent transmission of ovine cutaneous actinomycosis. Le Riche (1967) showed that dip washes containing 0.5% zinc sulphate or 0.1% magnesium fluosilicate would prevent the transmission of ovine cutaneous actinomycosis. Zinc sulphate is used in New Zealand as a dip bacteriostat where D. congolensis causes problems at dipping (Graham, pers. com. 1971).

VI. CONCLUSIONS

The literature on sheep dips and dipping is largely concerned with the control of arthropod ectoparasites of the skin and fleeces of sheep. These parasites have been a problem of sheep health for as long as records of the domestication of sheep have been kept. It is only within the last one hundred years however, that effective control of these organisms has been achieved.

During the last three decades, because greater efficiency of control of sheep arthropod ectoparasites has become possible, and because microbiological problems associated with sheep dipping have occurred, there has been a changing emphasis from entomological to microbiological considerations. It seems reasonable to speculate that this trend will continue and that more efficient antibacterial chemicals for the control of undesirable fleece micro-organisms will be sought.

CHAPTER IV

A REVIEW OF THE IN VITRO METHODS
OF TESTING ANTIBACTERIAL CHEMICALS

A great variety of techniques have been employed in the laboratory to study the effects of physical and chemical factors upon micro-organisms, since the role of microbes in the initiation of infection and disease was established in the late 19th century (Stuart, 1968). It is the purpose in this section to review some of the more commonly used methods for the in vitro evaluation of the sensitivity of bacteria to chemical compounds.

Bass and Stuart (1968) considered that an effective test method for antimicrobial materials must fulfil three basic requirements if useful information was to be obtained. Firstly, the test must yield data that can be interpreted accurately in terms of the practical antimicrobial situation. Secondly, it must be precise enough to give results that can be reproduced uniformly. Thirdly, it must be adequately controlled. To satisfy these requirements completely is difficult, but all tests must be considered critically against these criteria.

In many cases in vitro sensitivity tests are closely allied to procedures used in analytical microbiology, that branch of microbiology in which micro-organisms are used as reagents for the quantitative determination of chemical compounds (Gavin, 1956). This point was emphasized by Heatley (1949b) when he observed that almost any biological method of assay of an antibiotic could be adapted to measure

microbial sensitivity to the antibiotic. Much of the sophistication of sensitivity testing procedures has been developed by microbiological analysts, particularly those studying antibiotics, in response to the need for precision and accuracy in antibiotic assays. The methods of assay of antibiotics have been reviewed by Heatley (1949a) and Grove and Randall (1955), while more general reviews of analytical microbiology are those of Gavin (1956; 1957a and b; 1958 and 1959) and Kavanagh (1963).

Mention must also be made of the methods used to evaluate bacterial growth. Mallette (1969) and Postgate (1969) reviewed the procedures used for microbial growth evaluation. As anti-bacterial agent tests often involve the detection of a growth response by microorganisms to a chemical compound, modified growth evaluation methods can provide good sensitivity data.

The in vitro methods of testing the sensitivity of bacteria to antibacterial materials will be reviewed under the following headings:

- I. Direct methods
- II. Turbidimetric methods
- III. Agar Diffusion methods
- IV. Metabolic methods
- V. Other methods

Finally, some observations applicable to all methods of sensitivity testing will be made.

I. DIRECT METHODS

The direct methods of testing involve measurement of the reaction of microbial cells exposed to chemicals to show either effects on individuals or changes in cell numbers. Direct methods fall into four categories, namely: 1) microscopic methods 2) viable counting methods 3) measurements of colony diameter, and 4) measurement of dry weight of cells.

(1) Microscopic methods

Two approaches using microscopy have been used. In the first, individual cells may be counted, while in the second individual cells can be observed and changes in morphology recorded. A third possibility would be the continuous observation of living cells for antibacterial effects. However, Quesnell (1966) pointed out that methods of micro-culture present formidable difficulties, a fact that may account for the absence of reports of sensitivity tests where cells are maintained in a dynamic state under continuous observation.

(a) Microscopic counting:

Microscopic counting of bacterial cells is most often carried out using counting chambers containing known volumes of bacterial suspension that can be observed microscopically. Samples of a test culture containing known numbers of cells are exposed to the chemical. After a suitable period, the number of cells is again determined, and the level of test chemical that prevents growth of, or decreases the population can be determined. Haemocytometer slides (Brown and Winsley, 1971) and Thoma counting chambers (Hugo and Frier, 1969; Hugo and Bloomfield, 1971a) have been used in tests of antibacterial compounds.

The errors in microscopic counting of bacterial cultures can be large. Mallette (1969) cited the work of Norris and Powell who found that precision in counting depended on the investigator, the counting chamber, the organism being counted, and methods of filling the chamber, handling cell suspensions, and cleaning glassware. Also, inability to differentiate between dead and living cells and errors brought about by dilution may further decrease counting accuracy. The merits of direct microscopic counting for the determination of antibacterial effects are questionable.

(b) Microscopic Observations of Changes
in Cells:

Some workers have used direct observation of changes in cell morphology to indicate toxic levels of chemicals. Both light microscopy (Olitzki et al., 1967) and electron microscopy (Davies et al., 1968) have been used in sensitivity tests. However, these methods can only be used where the chemicals under test cause major morphological changes in the test organisms. Also, it is necessary to extend the test by other methods to determine whether or not the morphological changes observed correlate with antibacterial effects.

(2) Viable Counting Procedures

Postgate (1969) defined the viable count of a microbial population as the absolute concentration of viable organisms present. By determining the viable count of a test population before and after exposure to varied concentrations of a test chemical, it is possible to determine the concentration that inhibits population growth or reduces the number of viable cells, that is the levels of test compound that are respectively bacteriostatic or bactericidal to the test organism.

Postgate (1969) outlined the methods most commonly used to count the viable cells in bacterial populations, all of which are applicable to the testing of antibacterial agents. These include standard pour-plate methods, most probably number methods, drop plate methods, and membrane filtration counting methods. Duma and Warner (1969) described a method for viable counting which used Pasteur pipettes containing test organisms placed on agar containing test chemical.

The problems involved with viable counting procedures have been well documented (Postgate, 1969) and can be overcome by procedural modifications. The single factor that may cause the greatest inaccuracies is that of metabolic injury. Scheusner et al., (1971) outlined a viable counting method to determine the number dead and metabolically injured bacteria present after treatment with chemicals. Death of bacteria was determined by taking the difference in colony count obtained on an organic medium before and after exposure to test chemicals. Metabolic injury was determined by plating on an inorganic "minimal" medium. The difference between colony counts on the inorganic medium and the organic medium gave the numbers of bacteria that were metabolically injured after chemical treatment. Without modifications similar to this viable counting sensitivity data is of limited value, because of the importance of metabolic injury in the practical antimicrobial situation.

Despite this major drawback, viable counting methods have been popular for testing antibacterial compounds.

(3) Colony Diameter

Pirt (1966 and 1967) developed a model for the growth of bacterial colonies on the surfaces of solid media. He established that when the growth rate of an organism is varied by an inhibitor, other things being equal, the initial radial growth rate of a colony of that organism is inversely proportional to the doubling time.

Colony diameter measurements have been used to determine the effects of various compounds on bacteria (Turner and Jervis, 1968; Eroshin et al., 1968). Usually, test chemicals have been incorporated into solid media which were inoculated with indicator organisms. The rate of increase of colony diameter, or the colony diameter after a constant incubation period, may be measured, and differences in growth between treated organisms and those on control plates can be established.

Further evaluation of this method of bacterial growth assessment is required. However, it would appear that the approach is relatively simple, and if Pirt's (1966 and 1967) suggestions were correct, then the method could have considerable value.

(4) Dry Weight of Cells

Measurements of the dry weight of cells treated with test compounds compared with those of control treatments have been used to establish antibacterial sensitivity values (e.g. Turner and Jervis, 1968). However, the inherent errors of dry weight determinations (Mallette, 1969) make these procedures of limited value for sensitivity testing.

II. TURBIDIMETRIC METHODS

Clear liquid nutrient media, after inoculation with a number of viable bacteria and a period of incubation, become turbid due to light scattering at the cell surfaces. This is used as evidence of growth and multiplication of the bacterial population.

Turbidity of liquids can be determined either visually or with the aid of electronic instruments, usually spectrophotometers or nephelometers. The turbidimetric methods of sensitivity testing fall into two categories depending on the method of turbidity measurement used.

(1) Visual Turbidity Determination Methods

In these methods the response of an indicator organism is measured between two concentrations of a dilution series of a test chemical (Gavin, 1957b). A fixed volume of clear liquid culture medium is placed in a series of tubes. To these, dilutions of test chemical are added. A two-fold dilution series (Heatley, 1949b) has been commonly used, but dilution schemes giving narrower dilution steps have been proposed (e.g. Schmidt and Moyer, 1944). Each tube is inoculated with test organisms, and after incubation, the tubes are observed for growth as evidenced by the development of turbidity. The end-point, from which the concentration of chemical that just inhibits the test organism can be determined, lies between the concentration at which growth occurs and the concentration where there is no growth.

The degree of precision obtained in this type of test is dependent on the concentration of the test chemical in the dilution series; the narrower the increments, the greater the precision obtained (Gavin, 1957b). A number of difficulties may also be encountered. It

is essential for sterile conditions to apply throughout the procedure, as contaminants may produce turbidity that is confused with that of the test organism (Foster and Woodruff, 1943; Heatley, 1949b; Gavin, 1957b). Compounds that develop precipitates or emulsions when added to culture media cannot be tested (Gavin, 1957b). Difficulties may arise in end-point determinations. Arbitrary end-points are sometimes defined (Foster and Woodruff, 1943; Heatley, 1949a). All methods of visual end-point determination are subjective to a greater or lesser extent. Uneven susceptibility of individuals in the test population may give graded decreases in turbidity with changes in concentration making end-point determination difficult.

Despite the problems that do occur in tube dilution turbidimetric tests, they have been widely used to evaluate anti-bacterial compounds. The comparative simplicity of this type of test has made it popular.

Modifications of the tube dilution procedure have been proposed to increase the speed with which dilutions can be set up. Beargie et al., (1965) used spot-plates containing nine wells to accommodate a two-fold dilution series. Chitwood (1969) used plastic plates with ninety-six cups in a twelve by eight arrangement. With a 0.025 ml multi-microdiluter eight step two-fold dilution series for twelve antibiotics could be set up in less than one minute. Both of these micro-methods were shown to give results that correlated well with standard tube dilution tests and were more rapid and economical.

Although tube dilution turbidimetric methods only yield bacteriostatic concentration data for test chemicals, it is possible by using additional procedures, to obtain bactericidal sensitivity data. Heatley (1949b) described a procedure for this purpose. He

proposed that at the end of the turbidimetric test incubation period, a small inoculum from each tube in the series be subcultured onto solid nutrient medium. If test organisms grow in subculture from tubes showing no turbidity, then the concentration of test chemical in those tubes can be considered to be bacteriostatic. By noting the concentration of test chemical that yields little or no growth in subculture, bactericidal concentrations of test chemical may be determined.

Heatley (1949b) indicated that the main difficulties with this procedure were; firstly, the chance that the small inoculum used for subculture, usually one loopful, may not contain any viable cells that were present in the original culture; and secondly, that enough chemical may be carried to the subculture with the inoculum as to inhibit the test organism. Despite these drawbacks, subculturing of dilution series tubes has been a very commonly used method to establish bactericidal concentrations of antibacterial chemicals.

(2) Spectrophotometric Methods

Turbidity of a liquid bacterial culture exposed to an antibacterial agent either during or after an incubation period can be measured with a suitable electric photometer. A graded response to a test chemical can thus be obtained (Gavin, 1957b). Two types of spectrophotometric testing methods have been used; namely those where the overall change in turbidity after incubation is measured, and those that determine the effect of a chemical on the growth curve of a culture.

(a) Measurement of Overall Turbidity

Effects:

Foster (1942) outlined a method for the quantitative assay of penicillin, using a spectrophotometer to determine the effect of penicillin on Staphylococcus aureus (Oxford strain). This basic procedure has since been adapted for sensitivity testing by several authors. The indicator organisms were exposed to the test chemical in a broth medium and after an incubation period turbidity was measured and compared with that of untreated cultures.

(b) Measurement of Growth Curves:

Brown (1966) outlined a method which followed the growth curves of broth cultures of bacteria, and measured the effect of the addition of a test chemical to the culture medium. Broth was inoculated with test organisms and observations of absorbance of cultures were made at intervals during incubation. At a pre-determined absorbance value, when it had been found that cells were dividing exponentially, the anti-bacterial agent was introduced into the culture, and its effect on absorbance was recorded during further incubation.

A second approach was used by Mead (1969) and Neeman et al., (1970), whereby growth curves of organisms growing in liquid media containing test chemicals were measured spectrophotometrically. This method measured the increase in lag phase brought about by the chemical agent.

A third approach was used by Nagy et al., (1968) and Kari et al., (1971). Growth rates of test cultures treated with test chemicals and control cultures were measured and were converted into inhibition data thus:

$$100 \times \left[100 - \frac{\text{inhibition (\%)} = \text{growth of bacteria in presence of chemical}}{\text{growth of bacteria without chemical}} \right]$$

Spectrophotometric sensitivity tests suffer some of the drawbacks of visual turbidity tests. Chemicals that form suspensions or precipitates cannot be tested. Sterile conditions should be maintained, although this requirement is not critical because the generally shorter incubation periods employed may reduce the effect of contaminant organisms. Dead cells may cause turbidity that falsifies results. One of the main advantages of these tests is their rapidity. For example, Brown (1966) used incubation periods of no longer than five hours to test chemicals against Pseudomonas aeruginosa.

III. AGAR DIFFUSION METHODS

In these methods antibacterial chemicals, placed in a reservoir, diffuse out through a solid medium which has been inoculated with test bacteria. If the compounds under test are bacteriostatic or bactericidal to the indicator organism, a zone of inhibition of growth around the reservoir results after incubation (Gavin, 1957a). The effects of test compounds on the organisms are measured by the size of the inhibition zone produced. Usually these tests have been carried out in Petri plates, but culture tubes and larger rectangular plates have been used.

Two main methods of inoculation of solid media with test organisms have been used; namely, by spreading an inoculum over the surface of previously solidified medium or by seeding sterile liquid medium before it sets and allowing it to solidify. The second

approach has been more commonly used because it allows more accurate control of the test inoculum applied.

The major differences in agar diffusion methods occur in the type of chemical reservoir that have been used. The simplest approach, which was suggested by Beijerinck as early as 1889 (Heatley, 1949a), has been to apply a drop of test chemical to the inoculated agar surface. This method has not been commonly used however, because it is difficult to accurately control drop sizes. Wells cut into the solid medium have been used also. These may be either rectangular troughs (Fleming, 1929) or cylindrical holes cut with a cork borer (Heatley, 1949a). It has been suggested that the wells should be sealed with small amounts of liquid agar after they have been made (Siddique et al., 1965) to prevent capillary movement of chemical along the agar-plate interface. Cutting wells can be a time-consuming procedure, although methods have been suggested that help to overcome this difficulty (Bell and Grundy, 1968).

The use of reservoirs made from glass or stainless steel cylinders was suggested by Abraham et al., (1941) and Heatley (1944). These were widely used in quantitative assays for penicillin. However, cylinders can be cumbersome, and it is usually necessary to heat them before placing on the agar surface to obtain a good seal between reservoir and agar.

By far the most commonly used chemical reservoirs, particularly in recent years, have been absorbent paper discs. These were suggested as an alternative to cylinders for quantitative penicillin assays (Vincent and Vincent, 1944; Epstein et al., 1944) and for sensitivity tests (Lamana and Shapiro, 1943; Morley, 1945; Bondi et al., 1947), but it was not until Bauer et al., (1966) outlined a

standardized single disc antibiotic susceptibility test that paper discs became widely used in sensitivity testing.

The factors that may cause variation and errors in agar diffusion methods have been critically studied, probably because these methods have been of particular importance in quantitative antibiotic assays. Foster and Woodruff (1943), Schmidt and Moyer (1944), Gavin (1957a), and more recently Davis and Stout (1971a), have discussed the factors which may cause errors in these methods. The factors considered important by these authors were: agar characteristics, including agar depth, composition, pH and water content; inoculum concentration; incubation temperature; procedural timing, and preparation and application of test chemicals. Davis and Stout (1971b) described a procedure designed to overcome or allow for variables occurring in disc diffusion methods.

IV. METABOLIC RESPONSE METHODS

These methods measure the effects of test chemicals on metabolic functions of indicator organisms and by comparison with untreated organisms, the sensitivities of test bacteria are determined. They have been used particularly in analytical microbiology (Heatley, 1949a; Gavin, 1959). The responses that have been used include spore germination, bacterial luminescence, enzyme activity, haemolytic activity, respiration response, acid production, and reduction of nitrate to nitrite. All can theoretically be used to measure the antibacterial effects of chemical agents (Heatley, 1949b). In fact only methods measuring respiratory responses, pH changes and enzyme activity have been reported.

Metabolic response methods have the common drawback of being more or less specific in their requirements in test organism characteristics.

(1) Respiratory Response Methods

The uptake of oxygen or evolution of carbon dioxide may be used to assess the activity of organisms. The effects of chemical agents on bacteria can be determined by comparing respiration rates of organisms treated with chemicals, with those of untreated cultures. Respiration rates of test cultures have been measured manometrically, with the Warburg respirometer (Oh et al., 1967; Oh et al., 1968) and using oxygen electrodes (Trudgill et al., 1971).

(2) Measurement of pH Changes

Growth of bacteria may be accompanied by production or utilization of acidic or basic compounds. Therefore, growth may be detected by changes in pH of the culture medium.

The detection of pH changes in cultures of indicator organisms treated with chemicals and those of untreated cultures has been used in sensitivity tests. Both pH - meters (Mead, 1969; Keele et al., 1971) and indicators (Hamdy and Blanchard, 1970) have been used to test antibacterial chemicals.

(3) Measurement of Enzyme Activity

Measurement of the activity of bacterial enzymes of cultures treated with chemical agents, and comparison with the enzyme activity of untreated cultures, has been used to determine sensitivity of bacteria to antibacterial chemicals. Two approaches have been used

for sensitivity testing. In the first, activity of an enzyme system is measured by visual changes in a medium containing a specific substrate (Wolf and Bobalek, 1967; Freeman et al., 1968). In the second, the activity of specific enzymes is measured (Keele et al., 1971; Trudgill et al., 1971).

V. OTHER METHODS

There are a number of sensitivity testing methods that do not conveniently fall into the previously considered categories. These include:

1. Agar dilution methods
2. The gradient plate method
3. The replica plate method
4. Extinction data methods, and
5. Methods using radioisotopes

Only the first has been commonly used, and critical evaluation of the others has not been attempted.

(1) Agar Dilution Methods

These methods have been commonly used for sensitivity testing. Serial dilutions of test chemicals are incorporated into liquified agar medium, which is poured into a series of plates. After the medium has solidified, indicator organisms are streaked onto the agar surface. The lowest concentration of the test chemical that inhibits visual growth of test organisms can be determined after incubation.

Difficulties may arise in these methods with end-point determination. Also, these tests are considered to be less accurate

than broth dilution turbidimetric procedures (Heatley, 1949a). However, absolute sterility is not essential, non-aqueous solutions may be tested directly, and several organisms can be tested simultaneously (Heatley, 1949a). These advantages may account for the popularity of agar dilution methods.

Modifications of the basic agar dilution procedure have been proposed. For example Mahony and Chadwick (1965) microscopically observed culture dishes for the development or otherwise of micro-colonies of test organisms, thus reducing the duration of the test. Kayser and van der Ploeg (1965) inoculated agar dilution plates with known numbers of test organisms, and counted the numbers of survivors, thus combining viable counting with an agar dilution method.

(2) Gradient Plate Method

Szybalski, in a paper by Bryson and Szybalski (1952) described a method for the isolation of antibiotic resistant bacterial strains. Two layers of agar that were complementary in wedge-shaped cross-section, were poured into a culture dish. The bottom layer contained no test chemical and after it solidified, the top layer containing an appropriate concentration of test chemical was added. The test chemical diffused downward and became diluted in proportion to the ratio of the thickness of the agar layers, thus creating a uniform linear concentration gradient along one axis of the culture dish. By streaking one or more test cultures along this axis and allowing them to grow, the length of the growing streaks gave a direct measure of the inhibitory concentration of the test chemical.

Some workers (Bickel et al., 1967; Sinha, 1969) have reported bacterial sensitivity data obtained using Szybalski's gradient plate method.

(3) Replica Plating Method

Lederberg and Lederberg (1952) described a method by which a number of organisms could be transferred from one culture plate to several others. The method employed a sterile velveteen stamp, the same size as a culture dish, which was pressed onto a grown culture. The stamp was then transferred to several other plates containing sterile agar medium. The method was originally used for indirect selection of bacterial mutants, but it has been used to apply several indicator organisms to plates containing test chemicals (Jurd et al., 1971).

(4) Extinction Time Measurements

Berry and Bean (1954) described a method by which the time to render all individual cells in a test population inviable could be determined. The procedure exposed a test population to test compounds in a number of tubes, and at specified time intervals the test chemical was quenched. The culture tubes were then incubated for a period and observed for presence or absence of growth. In this way the mean death time values were determined from the results obtained from several replicates.

The method of Berry and Bean (1954) has been used by Hugo and Frier (1969), but the comparative unpopularity of the procedure could be due to the large numbers of tubes required for a single test.

(5) Methods Using Radioisotopes

Davies et al., (1968) used a radioisotope technique as a sensitivity test. Organisms were grown in a ^{32}P labelling medium. Portions of the labelled cultures were treated with test chemicals and, after centrifugation the cell-free supernatant was assayed for radioactivity. High levels of radioactivity of the supernatant indicated a high degree of release of intra-cellular components and a high level of antibacterial activity. A similar procedure was used by Hibbitt and Benians (1971).

Radioisotope methods have been more commonly used in mode of action studies (e.g. Kari et al., 1971; Trudgill et al., 1971), but it seems likely that radioisotopes may become more important in the assessment of antibacterial chemical agents.

VI. CHEMICAL CONSIDERATIONS APPLICABLE TO ALL IN VITRO TESTING METHODS

(1) Test Organisms

Indicator organisms are essential to all testing procedures for antibacterial chemicals. Test organisms most commonly used are those encountered in particular situations where control of bacterial growth is necessary. However, in many of the standard testing procedures developed for the evaluation of antimicrobial preparations (Lawrence and Block, 1968), standard organisms not necessarily found in practical situations are often used.

Gavin (1956) pointed out that controlled growth of the indicator organism is essential in any procedure. He also observed that a test can be affected by the age of the test culture, the size of inoculum, temperature, and oxygen relationships, and pH, water content and

chemical constituents of the medium used. These factors are important not only in achieving the desired measurable response from test organisms, but also in maintaining an acceptable level of accuracy and reproducibility. Furthermore, it is preferable for indicator organisms to be easily cultivated and to show invariable sensitivity to test chemicals.

(2) Test Chemicals

Materials developed as possible antibacterial agents vary widely in chemical and physical properties. Difficulties may arise when some compounds are tested using methods requiring specific characteristics in test chemicals. For example, emulsions cannot be tested using turbidimetric procedures.

(3) Reporting of Sensitivity Values

There are several ways in which sensitivity values have been reported. Different test procedures yield different types of sensitivity data, while some tests give more than one type of value. The sensitivity values most commonly used are now considered.

(a) Minimal Inhibitory Concentration: The minimal inhibitory concentration (MIC) is the concentration of a test chemical that just inhibits growth of an indicator organism, and is commonly used to express bacterial sensitivity to compounds.

(b) Minimal Bactericidal Concentration: The minimal bactericidal concentration (MBC) is the concentration of a test chemical that just kills the test organisms. MBC values can be obtained from viable counting and tube dilution turbidimetric procedures. MBC values may be applied in an arbitrary manner.

For example, most authors reporting tube dilution sub-culture results determine MBC values from the sub-cultures yielding no growth. Others, including Levitan (1967), Axelrod et al., (1971), Bodey and Stewart (1971), and Bodey and Deerpake (1971), defined the MBC end-point as that concentration of test chemical yielding less than ten colonies of test organism per subculture streak.

(c) Sensitivity Classifications:

Sensitivity data may be used to classify organisms as sensitive or resistant to test chemicals, or to express the effects of chemicals on test organisms. An arbitrary value in the data available has been used to delineate sensitivity categories. Bondi et al., (1947) and Bauer et al., (1966) classified the sensitivity of test organisms in terms of agar diffusion inhibition zone diameter. Kayser and van der Ploeg (1965) applied arbitrary levels to viable counting data to indicate "no inhibition", "partial inhibition" or "complete inhibition" of indicator organisms. Davies and Field (1969) processed viable counts graphically and estimated the concentrations of test chemicals which produced 10^{-1} , 10^{-2} , and 10^{-4} survivors.

Although this approach to reporting sensitivity values has often been used, the arbitrary way in which terms such as "bacteriostatic" and "resistant" have been applied may lead to confusion of interpretation between different sets of results.

(d) Comparison with Standard Organisms:

It is possible to express the sensitivity of organisms by comparing them with organisms of known sensitivity. For example Lacey and Boswell (1968) used the Oxford strain of Staphylococcus aureus (ACTC 6571) for comparison. Similarly, Jeffries (1968)

included several unspecified strains of known sensitivity to novobiocin and lysozyme in a test, and by comparison with these, classified members of the Micrococcaceae into sensitivity groups.

(e) Relative Inhibitory Indices:

Eroshin et al., (1968) expressed the effects of chemicals on test organisms in terms of a "comparative growth index" determined from colony diameter measurements. This index was given by the ratio:

$$\frac{\text{Rate of increase of colony size on experimental medium}}{\text{Rate of increase of colony size on normal medium}}$$

This type of treatment could be given to sensitivity data obtained from almost all types of test procedures.

(f) Mathematical Formulations:

Mather (1949) described a method of analysis of extinction time data which gave "single mean survivor time" values. This approach was suggested as an accurate statistical treatment for extinction time results by Berry and Bean (1954).

Schubert (1970) outlined a formulation by which cell growth inhibition by chemical or environmental agents could be expressed as a straight line. This approach, Schubert (1970) claimed, provided a simple, economic and consistent way of characterizing growth inhibitors and evaluating their possible modes of action.

VII. CONCLUSIONS

This review has outlined the methods most commonly used to establish in vitro sensitivity values of bacteria to chemical agents. The methods discussed differ in the ways that responses of organisms to chemicals are measured, in the types of sensitivity values they yield, and in their requirements in test chemicals and organisms. The procedures used for any study will be limited by these factors.

Any in vitro testing method designed should fulfil the requirements outlined by Bass and Stuart (1968) as completely as possible. In any case, sensitivity values should always be accompanied by precise descriptions of the procedures used to establish them, and the conditions under which they were obtained. This is of paramount importance, because sensitivity values may be profoundly influenced by experimental factors (Heatley, 1949b).

SECTION 2 EXPERIMENTAL

CHAPTER V

INTRODUCTION TO EXPERIMENTAL STUDIES

The aim of this study was to evaluate several chemicals as bacteriostatic agents for use in plunge sheep dips. Seven chemicals were evaluated; five were supplied by Ivon Watkins-Dow Limited, New Plymouth, one by Dr R.C. Close of Plant Diseases Division, D.S.I.R., Lincoln, and one by Tasman Vaccine Laboratories Limited, Upper Hutt.

The experimental programme consisted of two sections. A laboratory study was carried out using several in vitro methods of testing the chemicals for their ability to inhibit or kill a range of species of micro-organisms that may be of importance when associated with sheep dipping. The second section entailed a field evaluation of some of the test chemicals.

I. ORGANISMS

(1) Cultures

The test cultures were obtained from the sources indicated in Table 5.1, and the identity of each culture was checked. Samples of each culture were Gram stained, by the method of Harrigan and McCance (1966), and flagella stained by the method of Rhodes (1958).

Measurements of cells in Gram stained smears were made using an ocular micrometer at x 1000 magnification. Colony characteristics were examined with the aid of a binocular microscope at up to x 45 magnification.

TABLE 5.1 * Indicator cultures and their sources

Culture	Source
<u>Escherichia coli</u>	Culture collection at the Microbiology Department, Lincoln College, Canterbury, N.Z.
<u>Escherichia coli</u> (H ₂ S)	Wallaceville Animal Research Centre, Upper Hutt, N.Z.
<u>Pseudomonas aeruginosa</u>	Culture collection at the Microbiology Department, Lincoln College, Canterbury, N.Z.
<u>Erysipelothrix rhusiopathiae</u>	Wallaceville, Animal Research Centre, Upper Hutt, N.Z.
<u>Dermatophilus congolensis</u>	Culture collection of the Microbiology Department, Lincoln College, Canterbury, N.Z.

The cultures used were:

- (a) Escherichia coli (Migula) Castellani and Chalmers, 1919.

The culture of Escherichia coli used was obtained from the Lincoln College Microbiology Department culture collection. Cells of this strain were Gram-negative, non-motile rods, usually from 3.0 to 3.5 μm long, by 0.4 to 0.4 μm wide, and occurring singly or in pairs. Colonies on Brain Heart Infusion Agar were cream and non-spreading after 24 hours incubation at 37°. In liquid Brain Heart Infusion at 37°, a turbid, heavily sedimented culture developed after 24 hours.

E. coli was included in this study because of its possible pathogenicity to sheep, and also because this organism has been commonly used in tests for antibacterial chemicals.

- (b) A Sulphur Reducing Strain of Escherichia coli.

Blackening of dip washes, caused by sulphur reducing strains of bacteria, is one of the major problems associated with plunge dipping

(Graham, pers. com., 1971). The sulphur reducing strain of E. coli used in this study (E. coli (H₂S)) was obtained from Wallaceville Animal Research Centre, Upper Hutt. Cells of this culture were Gram-negative rods, 3.0 to 3.2 µm long by 0.4 to 0.5 µm wide. They were motile possessing peritrichous flagella. Brain Heart Infusion agar colonies were cream after 24 hours at 37°. Colonies, when grown for longer than 24 hours, rapidly spread over the agar surface of a streaked plate. In liquid Brain Heart Infusion an even, dense turbidity resulted after 24 hours at 37°.

(c) Pseudomonas aeruginosa (Schroeter) Migula 1900

A culture of Pseudomonas aeruginosa held in the culture collection of the Microbiology Department, Lincoln College, was used in this study. Cells of this culture were Gram-negative and were 1.4 to 1.6 µm long by 0.5 µm wide. The cells were motile, each possessing a single, polar flagellum. Brain Heart Infusion Agar colonies were large, greenish and spreading when the organism was grown at 37° for more than 24 hours. A green diffusible pigment was evident around the colonies after longer incubation. In liquid Brain Heart Infusion culture, growth was marked by heavy turbidity and a thick, greenish sediment after 24 hours at 37°.

Ps. aeruginosa is capable of causing various discolourations in fleece wool, as described in Chapter III.

(d) Erysipelothrix rhusiopathiae (Migula) Buchanan 1918.

The reporting of post-dipping lameness (McLean, 1948) and the isolation of Erysipelothrix rhusiopathiae as the organism involved (Whitten et al., 1948), was the first direct association of a sheep health problem with micro-organisms from sheep dipping baths. The culture of Er. rhusiopathiae used in this study was obtained from

Wallaceville Animal Research Centre, Upper Hutt. The cells of this culture were smooth, slender, non-motile, Gram-positive rods, 2.0 to 3.8 μ m long by 0.3 μ m wide. Brain Heart Infusion Agar colonies were small, greenish, and transparent after 48 hours incubation at 37°. Growth in liquid Brain Heart Infusion gave slight, mottled turbidity after 24 hours at 37°.

(e) Dermatophilus congolensis van Saceghem 1915 emend.
1916, 1934.

Dermatophilus congolensis, an actinomycete, is the causative organism of ovine cutaneous actinomycosis (Roberts, 1961). The culture used in this study was obtained from the Lincoln College Microbiology Department culture collection. This organism had a variable morphology, but cells were consistently Gram-positive. Branching filaments of about 1.0 μ m width and showing varying degrees of segmentation were commonly present in cultures. However, coccoid cells and motile "zoospores" also occurred with characteristics within the range of variability noted in Gordon's (1964) description of the organism. Agar (Roberts, 1961) colonies were of the umbinate, annelliform, or "cake crumb" types described by Gordon (1964) and were pale orange after 7 days incubation at 37°. In broth (Roberts, 1961) the organism produced a thick sediment, with a clear supernatant fluid and an adherent ring at the surface after 7 days at 37°. Sometimes small hard colonies developed, adhering to the wall of the culture tube beneath the surface of the broth.

(2) Growth Media

Four growth media were used for the culture of test organisms:

(a) Medium I. Brain Heart Infusion

Medium I (Oxoid Limited, London S.E. 1, England) contained: 12.5g/l calf brain infusion solids, 5.0g/l beef heart infusion solids, 10.0g/l proteose peptone, 5.0g/l sodium chloride, 2.0g/l dextrose, and 2.5g/l anhydrous disodium phosphate. The dry Brain Heart Infusion powder was dissolved in tap water and had a pH of 6.9 after autoclaving at about 121° for 20 minutes.

(b) Medium II. Brain Heart Infusion Agar

Medium II was made from Medium I with 10g/l Bacteriological Agar (Davis Gelatine N.Z. Ltd., Christchurch) added. The pH of this medium was 6.9 after autoclaving at about 121° for 20 minutes.

(c) Medium III

Medium III (Roberts, 1961) contained 5g/l Bacto beef extract, 10g/l proteose peptone, 1g/l Bacto yeast extract (all from Difco Laboratories, Detroit 1, Michigan, U.S.A.), 1.5g/l Analar D-glucose (B.D.H. Laboratory Chemicals Division, Poole, England) and 5 g/l sodium chloride (May and Baker Ltd., Dagenham, England) all dissolved in tap water. The pH of the broth was 6.8 after autoclaving at about 121° for 20 minutes.

(d) Medium IV

Medium IV (Roberts, 1961) was made from Medium III by the addition of Bacteriological Agar (Davis Gelatine N.Z. Ltd, Christchurch). The pH of this medium was 6.8 after autoclaving at about 121° for 20 minutes.

Medium III and IV were used for the culture of D. congolensis only. E. coli, E. coli (H₂S), Ps. aeruginosa and Er. rhusiopathiae were all cultured on Media I and II. Cultures were maintained on

slopes of Medium II and Medium IV (D. congolensis) in a refrigerator at 6°. Subcultures were made onto fresh slopes at approximately 8 week intervals, and after a period of incubation at 37° (7 days for D. congolensis and 48 hours for the other organisms), the subcultures were returned to the refrigerator.

(3) Standardization of Inoculum

It is important for the levels of inoculum used in in vitro tests to be recorded. In this study the inoculum of each organism was standardized for each test by the following procedures:

For E. coli, E. coli (H₂S), Ps. aeruginosa and Er. rhusiopathiae subcultures were made from stock slopes into 10 ml portions of sterile Medium I, and incubated for 24 hours at 37°. After incubation the optical densities of the cultures were standardized using sterile Medium I as the diluent. Optical density measurements were carried out at 540 nm on a prism spectrophotometer (Model DB, Beckman Instruments Inc., Scientific and Process Instruments Division, U.S.A.). Plate counts were carried out on the standardized cultures using Medium II with plates incubated at 37°. Colonies were counted after 24 hours incubation for E. coli, E. coli (H₂S) and Ps. aeruginosa, and after 48 hours for Er. rhusiopathiae. Optical densities and the corresponding inoculum concentrations are recorded in Table 5.2

TABLE 5.2 Inoculum concentrations and corresponding optical densities of standardized cultures of test organisms

Organism	Optical Density at 540 nm	Number of colony forming units per ml culture
<u>E. coli</u>	1.70	1.92×10^{10}
<u>E. coli</u> (H ₂ S)	1.30	2.01×10^{10}
<u>Ps. aeruginosa</u>	1.15	4.20×10^{10}
<u>Er. rhusiopathiae</u>	0.25	1.55×10^9

II. TEST CHEMICALS

The structural formulae of the chemicals used in this study are shown in Fig. 5.1. The compounds tested were:

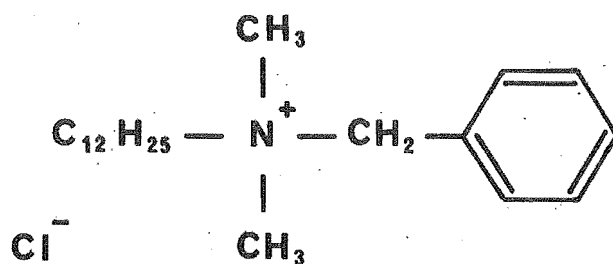
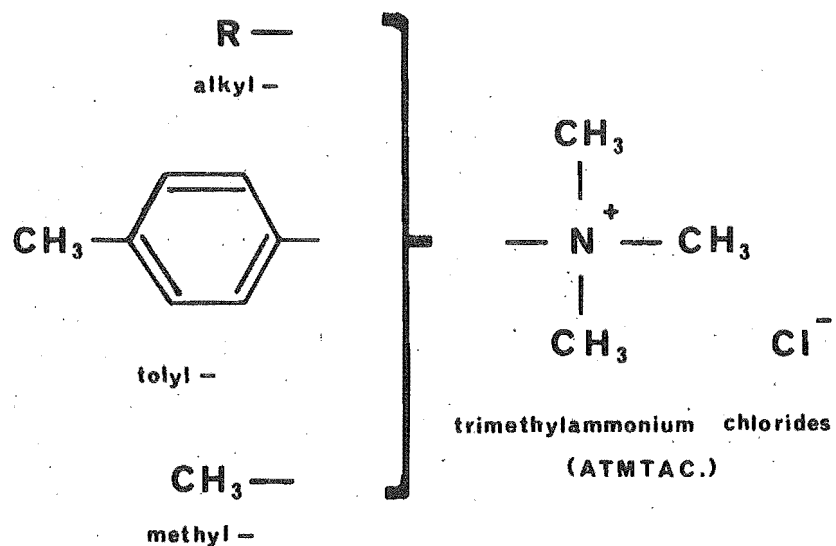
(1) A Mixture of Alkyl-, Toly-, and Methyl-, trimethylammonium Chlorides (ATMTAC)

This mixture of quaternary ammonium compounds was supplied by Farm Chemicals Company, Mapua, Nelson, through Dr R.C. Close, Plant Diseases Division, D.S.I.R., Lincoln, under the trade name "Hyacide 100." The preparation was a clear water soluble liquid containing 20% alkyl-, toly-, and methyl-, trimethylammonium chlorides. The mixture was recommended as a disinfectant-deodorant for poultry and industrial uses.

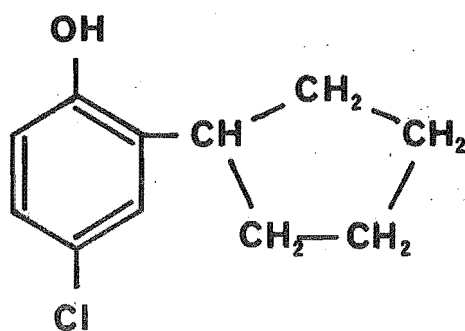
(2) Lauryldimethylbenzylammonium Chloride (LDBAC)

The second quaternary ammonium compound tested was supplied by Ivon Watkins-Dow Limited, New Plymouth, under the trade name "Aramin". The preparation was a clear viscous liquid containing 50% LDBAC. The material was soluble in water.

Fig. 5.1 Structural Formulae of Test Chemicals.

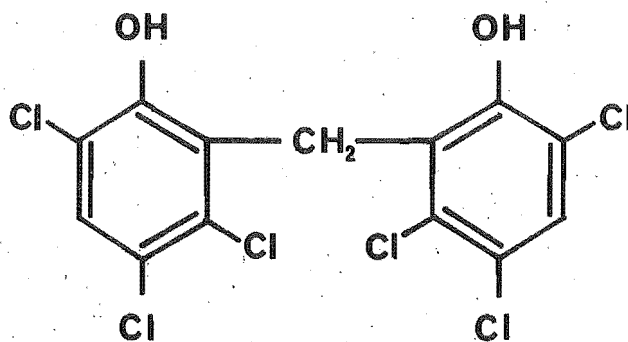


Lauryldimethylbenzylammonium chloride (LDBAC.)

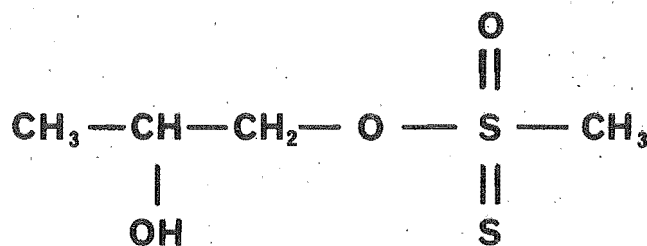


4-Chloro-2-cyclopentylphenol (CCPP.)

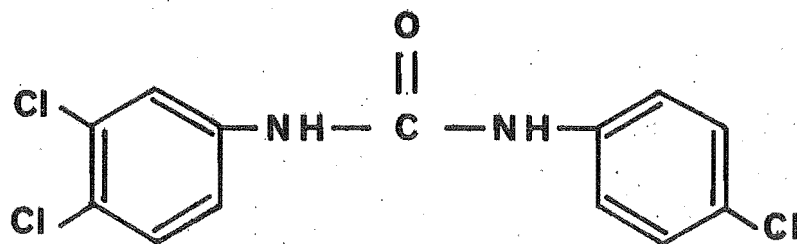
Fig. 5.1 Continued



2,2'-Dihydroxy-3,5,6,3',5',6'-hexachlorodiphenylmethane (HCP.)



2-Hydroxypropylmethanethiosulphonate (HPMTS.)



3,4,4'-Trichlorocarbantlilide (TCC.)

(3) 4-Chloro-2-cyclopentylphenol (CCPP)

CCPP is an aliphatic substituted derivative of chlorophenol. The antibacterial activity of this type of chemical has been established (Klarman et al., 1933). CCPP was supplied by Ivon Watkins-Dow Limited, New Plymouth, under the trade name "Dowicide 9". It was a clear golden viscous liquid, containing 96% CCPP, which was almost insoluble in water. The solvent used for this chemical was acetone, and emulsions were created using the emulsifier concentrate described below.

(4) 2,2'-Dihydroxy-3,5,6,3',5',6' - hexachloro-diphenylmethane (HCP)

This compound, known commonly as hexachlorophene, is a chlorinated methylene bis-phenol. It has been used widely in human skin degerming, skin deodorant, skin antiseptic and oral deodorant preparations, and as an additive to disinfectants and detergent sanitizers for over 25 years (Gump and Walter, 1968). The wide use of this chemical has been due to the fact that, unlike most other phenols, chlorine compounds and quaternary ammonium compounds, HCP possesses the rather unique property of retention of antibacterial activity in the presence of soaps (Joswick, 1961; Gump and Walter, 1968).

The HCP used in this study was supplied by Ivon Watkins-Dow Limited, New Plymouth, as a 100% pure free-flowing white powder. It was practically insoluble in water. Actone was used as the solvent in all tests, and emulsions were made using the emulsifier concentrate described below.

(5) 2-Hydroxypropylmethanethiosulphonate (HPMTS)

HPMTS is an aliphatic thiosulphonate which was been approved for use as a slimicide in the manufacture of food contact paper and paperboard in the U.S.A. (Anon, 1970). The chemical used in this

study was supplied by Ivon Watkins-Dow Limited, New Plymouth, as a clear viscous liquid containing 80% HPMTS in water. The compound was soluble in water.

(6) 3,4,4'-Trichlorocarbanilide (TCC)

TCC is a substituted diphenyl urea and has been used as a human skin germicide in soap preparations to control fungi and Gram-positive bacteria (Hodes and Stecker, 1968). It was supplied by Ivon Watkins-Dow Limited, New Plymouth, as pure TCC, a fine white powder which was almost insoluble in water. In this study the solvent used was acetone and emulsions were prepared using the emulsifier concentrate described below.

(7) Arsenical Bacteriostat

Arsenical sheep dips were unknowingly used as bacteriostats until the early 1940s, and since then arsenicals have been recommended as plunge dip bacteriostatic agents. For these reasons it was considered necessary to include an arsenical in this study for comparative purposes. The bacteriostatic preparation used was supplied by Tasman Vaccine Laboratories Limited, Upper Hutt. The bacteriostat was sodium arsenite, which is generally expressed in terms of arsenious oxide (As_2O_3). The preparation contained 80% As_2O_3 , and all concentrations are expressed in terms of this compound.

(8) Emulsifier Concentrate

An emulsifier concentrate was supplied by Ivon Watkins-Dow Limited, New Plymouth. It contained ethoxylated nonylphenol, ethoxylated poly(oxypropylene) and calcium dodecylbenzenesulphonate in unknown proportions. It was used as a 3% solution for the preparation of emulsions of CCPP, HCP, and TCC.

III METHODS OF TESTING

Three criteria were used to decide upon the types of in vitro test for the evaluation of the test chemicals. These were:

(1) Commonly used methods which have been assessed critically and adapted to give accurate results should be used;

(2) Availability of equipment would govern the choice of methods; and

(3) Methods differing in basic principles should be used so that a comparison of methods could be made. In fact, four in vitro tests were carried out. These were:

(1) A broth dilution turbidimetric and agar subculture method, which has been very commonly used and critically assessed. The method requires only simple equipment.

(2) An agar diffusion method, which has been used for quantitative assay and susceptibility tests of antibiotics. It has been critically evaluated and has simple requirements in equipment.

(3) A manometric method, which was relatively sophisticated, and has not been commonly used for susceptibility tests. However, a Warburg respirometer was available and a test was devised using this apparatus; and

(4) A gradient plate method which has not been commonly used but has the advantage of simplicity.

Not all the chemicals and test organisms were used in all the methods, because specific procedures had specific requirements in test materials.

A field trial was also carried out in which live sheep were treated with some of the test chemicals. The degree of correlation

between field and laboratory results was assessed, and from this the value of the in vitro tests used was determined.

CHAPTER VI

BROTH DILUTION TURBIDIMETRIC AND

AGAR SUBCULTURE METHOD

As mentioned previously, the broth dilution turbidimetric method of sensitivity testing has been widely used. The method has remained essentially the same for a long period (Fleming, 1929; Traub, 1970), and remains one of the simplest procedures available to assess the sensitivity of bacteria to test chemicals. Broth dilution techniques have been critically examined to determine the factors that decrease their precision and accuracy by Foster and Woodruff (1943), Schmidt and Moyer (1944), and Gavin (1957b).

Broth dilution methods determine levels of chemicals that are bacteriostatic to test organisms. However, by further simple techniques, these procedures can be extended to provide information on the bactericidal concentrations of chemicals.

In this study, three of the seven chemicals to be evaluated were insoluble in water, and therefore could not be tested using visually detected turbidity as an indication of bacterial growth. Nevertheless, by incorporating a subculturing procedure into the test, antibacterial information could still be gained for these chemicals from the basic dilution technique. As Dermatophilus congolensis does not form turbid broth cultures, this organism could not be tested using this method.

I. MATERIALS AND METHODS

(1) Organisms

Escherichia coli, E. coli (H₂S), Pseudomonas aeruginosa, and Erysipelothrix rhusiopathiae were subcultured from stock slopes of Medium II into 10ml aliquots of sterile Medium I in test tubes with aluminium caps (Oxoid Limited, London S.E. 1, England), and incubated for 24 hours at 37°. From these subcultures further transfers were made to the same medium, and these were incubated for 24 hours at 37°. Turbidity of the second subcultures was adjusted to give the standard inoculum for each organism, as set out in Chapter V.

(2) Chemicals

The seven test chemicals were prepared as solutions or emulsions with sterile distilled water to give a concentration of 10,000 µg/ml.

(3) Procedure

For each combination of test chemical and test organism, two replicate dilution series, each in thirteen tubes, were made up. Previously sterilized 16 x 150 mm rimless test-tubes, each with an aluminium cap, were filled under aseptic conditions, with 10 ml of sterile Medium I using a previously sterilized continuous pipetting apparatus (Becton, Dickinson and Company, Rutherford, New Jersey, U.S.A. Catalogue number 1271). The tubes were arranged into groups of thirteen in test tube racks. In eleven of these tubes a two-fold dilution series of test chemical was prepared by the following procedure:

Ten ml of a 10,000 µg/ml solution or emulsion of test chemical, or 10 ml of sterile water or 3% emulsifier for controls,

were placed in the first tube of the series using sterile blow-out pipettes. After mixing, 10 ml of the broth-chemical mixture were transferred into the second tube and mixed. This procedure was repeated for each of the tubes. From the eleventh tube in each series 10 ml of broth-chemical mixture were discarded. The remaining two tubes were used as controls containing no chemicals. This procedure gave a dilution series of the chemical of 5000, 2500, 625, 312, 156, 78, 39, 19, 9.5, and 4.8 $\mu\text{g/ml}$.

Each tube, except for one of the controls, was then inoculated with test organisms. One loopful of standardized test culture was placed into each tube. A wire loop made from 0.5mm diameter wire, 9 cm long and bent at 45° to the handle, was used for this. The loop was of 2.3 mm internal diameter, and delivered a volume of 0.002 ml, as calibrated by removing five hundred loopfuls of Medium I from a 5 ml measuring cylinder. One loopful of the test cultures gave an inoculum in each tube corresponding to approximately 4×10^7 colony forming units (cfu) of E. coli, 4×10^7 cfu E. coli (H_2S), 8×10^7 cfu of Ps. aeruginosa, and 3×10^6 cfu of Er. rhusiopathiae.

Once inoculation of all tubes was completed, tubes were placed into a shaking waterbath (Manufacturing Laboratory Supplies Limited, Lower Hutt. Catalogue number QC. 350), and incubated at 37° for 24 hours. The shaking system on the water bath was set to oscillate at approximately 130 oscillations per minute, but the reliability of the model used was suspect and some variation from this rate was experienced. After incubation, tubes containing water soluble test chemicals were observed for the presence or absence of growth of the test organisms.

All tubes were subcultured onto Medium II in Petri plates. The thirteen tubes in each series were streaked onto three plates, two of the plates accommodating streaks from four tubes, the other from the remaining five. Subculture plates were then inverted and incubated at 37° for 24 hours, when the streaks were examined, and the presence or absence of colonies noted.

The procedure described gave a tube dilution turbidimetric and subculture test for water soluble test chemicals, and a tube dilution and subculture test for water insoluble compounds.

II. RESULTS

For water soluble test chemicals, the presence or absence of growth of test organisms was determined by visual inspection of dilution tubes for presence or absence of turbidity. Culture tubes were stirred with a mechanical test-tube stirrer for about 5 seconds before they were observed. Turbidity of the broth was recorded as heavy, slight, or nil. These results are shown in Appendix 1, Table A1.1

For all test compounds, subculture plates were examined, and the growth on each streak was recorded as heavy, medium, slight or nil. These results are shown in Appendix 1, Table A1.2.

From these results, bacteriostatic concentrations of the water soluble compounds, and bactericidal concentrations of all the chemicals were determined. The minimal inhibitory concentrations (MIC) of the water soluble chemicals were taken as the lowest concentration completely inhibiting growth in broth culture. The minimal bactericidal concentrations (MBC) of all chemicals were taken as the lowest concentration that killed the test organisms, as

indicated by their inability to grow on subculture. MIC and MBC values are set out in Table 6.1.

All the chemicals tested except TCC showed antibacterial activity against the test organisms, within the range of concentrations tested. CCPP was the most effective chemical against E. coli. HPMTS, ATMTAC, and LDBAC were less effective than CCPP, but all showed greater activity against this organism than did arsenic. HCP and TCC were both less effective than arsenic.

Arsenic was the most effective chemical in controlling E. coli (H_2S), with HPMTS, LDBAC, and ATMTAC showing intermediate activity. CCPP, HCP and TCC showed little or no antibacterial activity against this organism within the range of concentrations tested.

Against Ps. aeruginosa, arsenic was slightly more effective than HPMTS. The other test chemicals showed only limited or no antibacterial activity against this organism. This method showed Ps. aeruginosa to be the most resistant of the organisms to the test compounds.

Er. rhusiopathiae appeared to be the most sensitive of the organisms tested, but this could be a reflection of the smaller inoculum of this organism used per tube. The quaternary ammonium compounds ATMTAC and LDBAC were the most effective, and showed greater antibacterial activity than arsenic did against this organism. HPMTS and HCP, though less effective than arsenic, showed reasonable antibacterial activity. CCPP and TCC were not effective at the concentrations tested.

Overall, HPMTS and arsenic were the most effective of the antibacterial agents tested. The quaternary ammonium compounds were of intermediate effectiveness by comparison, and CCPP, HCP, and TCC were least effective against the range of test organisms.

TABLE 6.1 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of test chemicals expressed in μ g/ml as established using the tube dilution turbidimetric and subculture procedure.

> = Greater than

- = Not established by this procedure

Organism	Chemical	MIC	MBC
<u>E. coli</u>	Arsenic	1250	5000
	HPMTS	39	312
	ATMTAC	78	312
	LDBAC	78	625
	CCPP	-	156
	HCP	-	> 5000
	TCC	-	> 5000
<u>E. coli</u> (H ₂ S)	Arsenic	19	156
	HPMTS	156	625
	ATMTAC	625	2500
	LDBAC	625	1250
	CCPP	-	> 5000
	HCP	-	> 5000
	TCC	-	> 5000
<u>Ps. aeruginosa</u>	Arsenic	625	2500
	HPMTS	1250	2500
	ATMTAC	2500	5000
	LDBAC	1250	5000
	CCPP	-	> 5000
	HCP	-	5000
	TCC	-	> 5000
<u>Er. rhusiopath.</u>	Arsenic	39	156
	HPMTS	78	312
	ATMTAC	19	78
	LDBAC	19	78
	CCPP	-	> 5000
	HCP	-	625
	TCC	-	> 5000

CHAPTER VII

AGAR DIFFUSION METHOD

Agar diffusion methods, particularly those using absorbant paper discs as reservoirs, have been widely used in micro-bioassays for antibiotics. The factors that influence the accuracy and variability of disc diffusion assays have been studied and elucidated by a number of authors, including Foster and Woodruff (1943), Schmidt and Moyer (1944), Heatley (1949a), Gavin (1957a) and Davis and Stout (1971a).

Davis and Stout (1971b) outlined a disc plate agar diffusion procedure for microbiological antibiotic assays which allowed for or overcame the factors shown previously (Davis and Stout, 1971) to cause variability and error in these assays. The method used in this investigation was based on the assay procedure of Davis and Stout (1971b).

The method was used successfully with all the test organisms and chemicals. The procedure used incorporated a test to indicate the variability that occurred between agar plates, so that the effects of the modifications made to the Davis-Stout procedure could be evaluated.

I. MATERIALS AND METHODS

(1) Organisms

Escherichia coli, E. coli (H₂S), Psuedomonas aeruginosa, and Erysipelothrix rhusiopathiae were all subcultured from refrigerated stock Medium II slopes into culture bottles containing 10 ml of sterile Medium I. The subcultures were incubated for

24 hours at 37°, and were adjusted to the standard absorbance values (Chapter V) using sterile Medium I as a diluent.

Dermatophilus congolensis was subcultured from refrigerated stock Medium IV slopes into 10 ml portions of sterile Medium III in culture bottles containing glass beads. After 7 days incubation at 37°, the cultures were vigorously shaken for about 2 minutes to break up the filaments.

(2) Chemicals

Test chemicals were prepared as solutions to a concentration of 6000 µg/ml. Sterile distilled water was used as the solvent for arsenic, HPMTS, ATMTAC and LDBAC, while 65% acetone was used for CCP, HCP and TCC. The test solutions were stored in sealed 200ml bottles at room temperature.

(3) Procedure

The procedure used was taken almost exactly from the paper of Davis and Stout (1971b). However, whereas the Davis-Stout procedure was for microbiological assay, the technique used in this study was a sensitivity test so that only a very few concentrations of test chemicals were used. In most cases a single concentration was enough to make comparisons between test chemicals. Otherwise the procedure differed in the temperature at which plates were poured, and in the types of equipment used, including Petri plates, micro-syringes, the continuous pipetting apparatus and absorbant paper pads.

Aliquots of 200 ml of sterile Medium II (or Medium IV for D. congolensis) held at 45° in a water bath. (Manufacturing Laboratory Supplies, Limited, Lower Hutt, Catalogue number Q.C. 350) were seeded with 10 ml of standardized cultures of test organisms.

Thus, the standard inoculum for each organism was diluted to 1:20. The seeded liquid agar was poured into disposable plastic Petri plates (Tasman Vaccine Laboratory Limited, Upper Hutt) of 9.0 cm diameter. These plates were used in preference to glass ones because of their uniformity. Plates which did not have flat bottoms could not be used, as variation in agar thickness has been shown to cause considerable variations in disc diffusion tests (Davis and Stout, 1971a). An 8.0 ml quantity of seeded medium was poured into each plate using a continuous pipetting apparatus (Becton, Dickinson and Company, Rutherford, New Jersey, U.S.A. Catalogue number 3056). This volume gave an average agar thickness of 1.3 mm determined by measurement with vernier callipers. As each plate was poured, it was gently rotated on a level bench top to assure a flat and uniform agar layer was obtained in each plate.

The temperature at which agar was delivered into the plates was kept constant by pumping sterile water, held at 45° in the same water bath as the molten agar, through the continuous pipetting apparatus for twenty deliveries. The intake tube of the pipette was transferred to the seeded agar, and after discarding five deliveries of agar, filling of plates was commenced. This procedure maintained the agar at a constant temperature throughout the plate pouring procedure, as recommended by Davis and Stout (1971a).

After pouring, plates were left on the bench top for 20 minutes to cool. They were then inverted and stacked in the order in which they had been poured.

The plates were then set with absorbant paper pads. Plates were taken from the stack in order of pouring and placed over a guide. The guide was made from white paper on which two circles, one

with the same diameter as a Petri plate, the other with the same centre as the first but with a radius of 25 mm, had been drawn. Six marks had been made at 25 mm spacings on the smaller circle to indicate positions at which six absorbant paper pads were to be placed on the agar surface. The paper pads were cut from Whatman thick seed test paper (W. and R. Balston Limited, England) with a paper punch and were 6.2 mm in diameter.

After six pads had been placed onto a plate with a dissecting needle, 20 μ l of test chemical was applied to each pad with a 100 μ l tuberculin syringe (Hamilton Company Inc., Whittier, California, U.S.A. Catalogue number 710N). One chemical was applied to the 6 replicate pads in one plate so that with plates for distilled water and 65% acetone controls, nine plates were used to test all the chemicals against one organism.

After setting, the plates were put into a water jacket incubator at 37°. The plates were in the inverted position in pairs on one shelf of the incubator so that all plates were incubated at as near a constant temperature as was possible. The incubation period for E. coli, E. coli (H₂S) and Ps. aeruginosa was 24 hours and for Er. rhusiopathiae and D. congolensis 48 hours. After these periods, the plates were observed with the aid of a Quebec dark field colony counter (Model 3329, American Optical Corporation, New York, U.S.A.). The diameters of the zones of inhibition around pads were measured with vernier callipers.

II. RESULTS

Before the main test was carried out, a variation check was performed. This was done to determine the degree of variation between plates, brought about by modifications to the Davis and Stout (1971b) procedure. Seven plates seeded with D. congolensis were poured and set, and 120 μ g ATMTAC, were applied to each paper pad in each dish. The plates were incubated for 48 hours and the inhibition zone diameters were measured. The results of this test and statistical analysis of these are shown in Table 7.1. The between plate variation was shown to be insignificant within 1% confidence limits.

The mean zone diameters and statistical analyses of results are shown in Tables 7.2, 7.3 and 7.4, and in Appendix 2, Tables A2.4, A2.5, A2.6, A2.7 and A2.8. The zone diameter measurements are given in Appendix 2, Tables A2.1, A2.2, and A2.3. Where 120 μ g/pad gave inhibition zones greater than 40 mm, the test chemicals were applied to pads at lower concentrations to determine the levels at which measurable inhibition zones could be obtained. Some examples of inhibition zones are shown in Fig. 7.1.

Against E. coli, CCPP was the most inhibitory chemical, with HPMTS, LDBAC, ATMTAC and arsenic all showing similar effectiveness, but being less inhibitory than CCPP. HCP was still less anti-bacterial, while TCC was non-inhibitory at 120 μ g. All treatments except TCC and the controls showed degrees of inhibition that were significantly different within 1% confidence limits.

Arsenic was by far the most inhibitory chemical for E. coli (H_2S). HPMTS was much less inhibitory, but was more so than were ATMTAC, LDBAC, CCPP and HCP. The difference between CCPP and HCP

TABLE 7.1 Zone diameters (mm) from test to determine variation between plates. ATMTAC (120 μ g/pad) against D. congolensis.

Disc. No.	Plate Number							\bar{X}	S	S%
	1	2	3	4	5	6	7			
1	22.9	23.2	23.5	23.5	23.3	23.2	23.3	23.27	0.1939	0.83
2	23.5	23.0	23.2	23.1	23.7	23.1	23.0	23.23	0.2463	1.06
3	23.4	23.3	22.8	23.1	23.4	23.1	23.0	23.16	0.2107	0.91
4	23.6	23.7	23.1	23.3	23.2	23.3	23.0	23.31	0.2340	1.00
5	23.8	23.4	23.0	23.2	23.2	23.5	23.1	23.31	0.2517	1.08
6	23.4	23.2	23.1	23.3	22.9	23.0	23.4	23.19	0.1972	0.85

\bar{X} = Mean zone diameter

S = Standard deviation

S% = Coefficient of variation

TABLE 7.2

Mean zone diameters (mm) for test chemicals at 120 µg/pad against the test organisms.

Organism	Chemical	\bar{X}	S	S%
<u>E. coli</u>	Arsenic	10.52	0.2898	2.75
	HPMTS	13.87	0.2336	1.68
	ATMTAC	11.02	0.2898	2.63
	LDBAC	12.42	0.3171	2.55
	CCPP	16.82	0.3419	2.03
	HCP	8.62	0.1039	1.21
	TCC	6.20	0.0000	0.00
	Water	6.20	0.0000	0.00
	65% Acet.	6.20	0.0000	0.00
<u>E. coli</u> (H ₂ S)	Arsenic	> 40		
	HPMTS	13.23	0.3159	2.39
	ATMTAC	9.73	0.1135	1.17
	LDBAC	8.77	0.2042	2.33
	CCPP	7.32	0.0648	0.89
	HCP	7.20	0.0818	1.14
	TCC	6.20	0.0000	0.00
	Water	6.20	0.0000	0.00
	65% Acet.	6.20	0.0000	0.00
<u>Ps. aeruginosa</u>	Arsenic	15.82	0.5036	3.18
	HPMTS	10.52	0.1843	1.75
	ATMTAC	8.60	0.1292	1.50
	LDBAC	8.05	0.1256	1.56
	CCPP	6.20	0.0000	0.00
	HCP	7.72	0.1322	1.71
	TCC	6.20	0.0000	0.00
	Water	6.20	0.0000	0.00
	65% Acet.	6.20	0.0000	0.00
<u>Er. rhusiopathiae</u>	Arsenic	18.75	0.2630	1.40
	HPMTS	17.35	0.2218	1.28
	ATMTAC	21.12	0.4932	2.34
	LDBAC	23.13	0.3421	1.48
	CCPP	6.20	0.0000	0.00
	HCP	20.25	0.3404	1.68
	TCC	6.20	0.0000	0.00
	Water	6.20	0.0000	0.00
	65% Acet.	6.20	0.0000	0.00
<u>D. congolensis</u>	Arsenic	16.18	0.2433	1.46
	HPMTS	23.30	0.5802	2.49
	ATMTAC	23.13	0.1926	0.83
	LDBAC	24.67	0.2952	1.20
	CCPP	> 40		
	HCP	18.37	0.2330	1.27
	TCC	8.80	0.1526	1.73
	Water	6.20	0.0000	0.00
	65% Acet.	6.20	0.0000	0.00

 \bar{X} = Mean zone diameter (mm.)

S = Standard deviation

S% = Coefficient of variation

> = Greater than

TABLE 7.3 Mean zone diameters for CCPP against D. congolensis.

Amount of Chemical Per Pad	\bar{X}	S	S%
20 μ g	16.07	0.2539	1.58
10 μ g	11.37	0.1462	1.29
65% Acetone	6.20	0.0000	0.00

\bar{X} = Mean zone diameter (mm.)

S = Standard deviation

S% = Coefficient of variation

TABLE 7.4 Mean zone diameters for Arsenic Against E. coli (H_2S).

Amount of Chemical Per Pad	\bar{X}	S	S%
5 μ g	19.32	0.3111	1.61
Water	6.20	0.0000	0.00

\bar{X} = Mean zone diameter (mm.)

S = Standard deviation

S% = Coefficient of variation

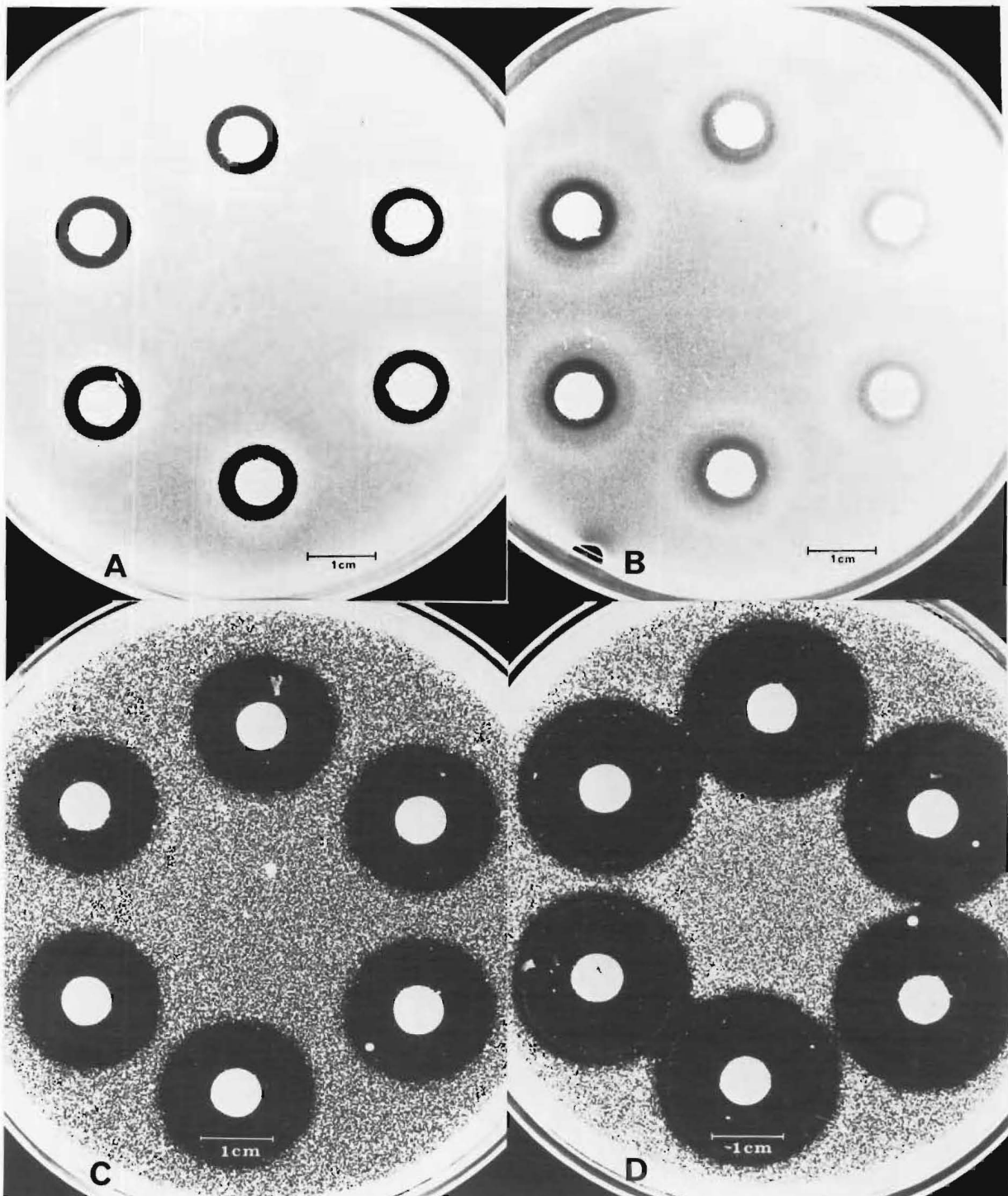


Fig. 7.1 Examples of Agar Diffusion Plates

- A** HPMTS against *Ps. aeruginosa*
- B** Arsenic against *E. coli*
- C** ATMTAC against *D. congolensis*
- D** LDBAC against *D. congolensis*

was insignificant at the 1% level of confidence. TCC gave no inhibition of E. coli (H_2S) at the concentration tested.

Ps. aeruginosa appeared to be the most resistant of the test organisms, with only arsenic showing any substantial degree of inhibition. LDBAC and HCP showed levels of inhibition that were insignificantly different within 1% confidence limits but were significantly different at the 5% level. TCC showed no greater inhibition at $120\mu g$ than did the controls.

Er. rhusiopathiae was inhibited markedly by LDBAC, ATMTAC, HCP, arsenic and HPMTS. Both CAPP and TCC showed no inhibition of this organism at the concentration tested.

D. congolensis appeared to be the most sensitive organism to the test chemicals. All the chemicals except TCC showed a high degree of inhibition. Even TCC showed a slight antibacterial effect. The degree of inhibition brought about by HPMTS and ATMTAC was insignificantly different within 1% limits of confidence.

III. SOME OBSERVATIONS

The diffusion plates were photographed using an enlarger with the plate taking the place of a photographic negative. The image thus obtained was exposed onto photographic paper, which was then processed in the normal way. Using this procedure permanent 2.5 times enlargements of the agar plates, showing inhibition zones, were obtained. On close study of these enlargements, variations in the form of inhibition zones and the presence of zones of stimulation were noted.

Two forms of inhibition zones were found. The most common showed zone edges that were either indistinct (slightly "furry") as in Fig. 7.2A, or showed a ring of slight growth some distance into the otherwise clear zone, as in Fig. 7.2D. Sokolski and Carpenter (1959) postulated that as a decreasing test chemical concentration gradient developed outwards from the paper pad, it seemed likely that indistinct zone borders could indicate a variation of susceptibility among the population of test cells. Bryson and Szybalski (1952) made the suggestion that gradual inhibition along a concentration gradient indicated that the toxic agent interfered with a simple chemical reaction of a low kinetic order, whereas abrupt inhibition indicated that a mathematically high order reaction, a chain of consecutive reactions for example, was interfered with.

The second type of inhibition zone (Fig. 7.2C) had a very distinct, sharp border. HPMTS produced this type of zone with all the test organisms except Ps. aeruginosa. It is possible that the Bryson and Szybalski (1952) explanation for abrupt inhibition is applicable for this chemical.

The occurrence of an area of stimulation, either immediately outside the inhibition zone, or further out, was universal for all chemicals and all test organisms. These stimulation zones are an expression of the phenomenon of hormoligosis, which was defined by Luckey (1959) as the stimulatory effect of very small amounts of an agent upon living organisms. Hormoligosis occurs with all types of living cells in response to all chemical and physical agents. A more specific phenomenon was described by Southam and Ehrlich (1943), who proposed the term hormesis (adj. hormetic) to designate a stimulatory effect of subinhibitory concentrations of any toxic substance on any organism.

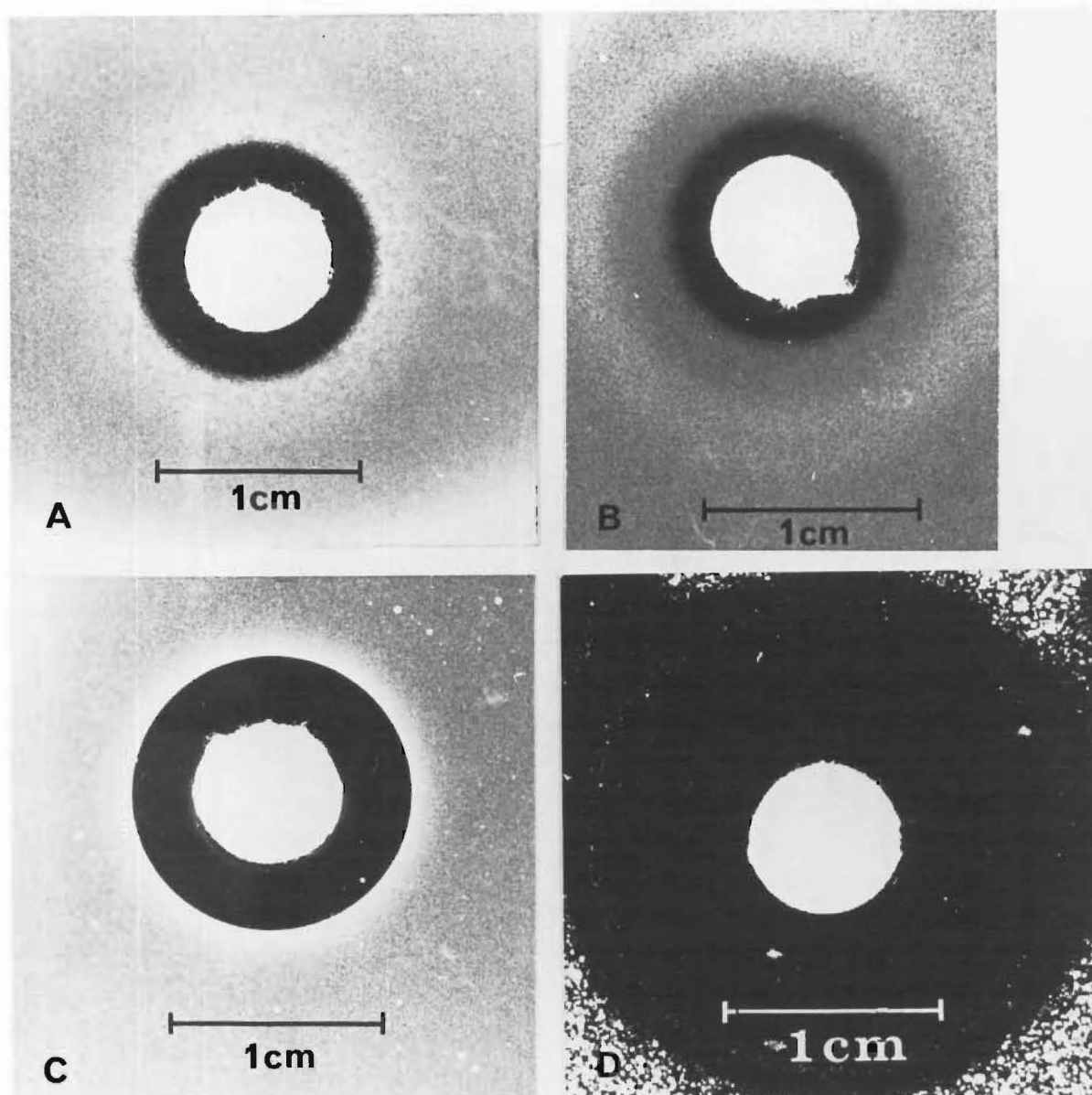


Fig. 7.2 Types of Inhibition Zone

- A** Zone with indistinct outer margin and adjacent stimulation zone. HPMTS with Ps. aeruginosa
- B** Zone with stimulation zone some distance outside zone margin. Arsenic with E. coli
- C** Zone with sharp outer margin and zone of stimulation. HPMTS with E. coli
- D** Zone with inner ring of growth. LDBAC with D. congolensis

Hormetic effects on bacteria have been described for both inorganic (Greaves, 1931; Shorb et al., 1954; Feeney et al., 1957) and organic compounds (Lamana, 1942; Miller et al., 1945; Eriksen, 1946; Pratt and Dufrenoy, 1947) and have been shown to occur both in vitro and in vivo (Stansfeld et al., 1944; Welch et al., 1946; Randall et al., 1947).

The hypotheses put forward to explain hormetic responses have been varied. Luckey (1959) noted that stimulation of bacteria around agar diffusion inhibition zones had been ascribed to an increased availability of nutrients diffusing out from inhibition zones where there were no organisms utilizing the constituents of the growth medium. A second explanation (Dufrenoy and Pratt, 1947; Pratt and Dufrenoy, 1947), was that stimulatory metabolites or growth factors were released by cells undergoing lysis, and these became available to the organisms growing in the immediate vicinity, but where concentrations of the lysogenic agent were not great enough to inhibit growth.

While these two explanations may be valid for hormetic responses in some agar diffusion tests, they do not explain the same phenomenon occurring in dilution tubes of antibacterial chemicals (Miller et al., 1945) or in agar plates containing standard concentrations of antibacterial compounds (Garrod, 1951). Furthermore, as shown in Fig. 7.2B, the zone of stimulation need not be immediately adjacent to the zone of inhibition. This type of stimulation occurred with arsenic, CCPP, LDBAC, and ATMTAC against E. coli, suggesting that this type of stimulation may be of a specific nature.

Hormetic phenomena must be a result of more complex interactions. It has been shown that cells experiencing poor temperature

or nutritional conditions, or from older cultures (Miller et al., 1945; Garrod, 1951) were stimulated more by subinhibitory levels of antibacterial chemicals than those experiencing optimum conditions. These observations alone suggest that hormetic responses are governed by more than nutrient availability.

Luckey (1959) made several suggestions attempting to explain stimulatory effects on cells of subinhibitory levels of chemicals. All involved activities at the sub-cellular level, and were concerned with enzyme production or enzyme activity. It seems reasonable that if stimulatory effects on bacteria are expressed as a shortening of generation times with a resulting increase in cell numbers, as was shown to be the case by Miller et al., (1945), then the effects must be due to the stimulation of a number of complex biochemical events that result in growth and cell division. Until the actual modes of stimulation are elucidated, and these may be different for the responses of each organism to each toxic agent, little can be gained from further speculation.

CHAPTER VIII

MANOMETRIC METHOD

Reports of the use of manometric techniques to compare the effects of toxic chemicals on bacteria have been relatively limited. This may be due to the fact that manometry requires sophisticated and therefore expensive equipment, particularly where it is used to measure respiration rates of micro-organisms. Nevertheless, some investigators, including Oh et al., (1967) and Oh et al., (1968), have applied manometry to the study of antimicrobial chemical agents.

The manometric technique employed in this study used the Warburg constant volume respirometer. This apparatus uses the principle that at constant gas volume, any changes in the amount of a gas can be measured by changes in its pressure (Umbreit, 1972). Using this instrument, the uptake of oxygen by actively growing cultures of indicator organisms was measured, and the effect of the test chemicals on oxygen uptake was determined. The method used was based on that outlined by Umbreit (1972).

I. MATERIALS AND METHODS

(1) Cultures:

Cultures of Escherichia coli, E. coli (H₂S), and Pseudomonas aeruginosa were prepared in the way described in Chapter V, and were standardized spectrophotometrically using sterile Medium I as the diluent. It was necessary to concentrate cultures of Erysipelothrix rhusiopathiae and Dermatophilus congolensis

because the oxygen uptakes of unconcentrated cultures of these two organisms were not rapid enough to give a quick test for the antibacterial activity of the chemicals being evaluated.

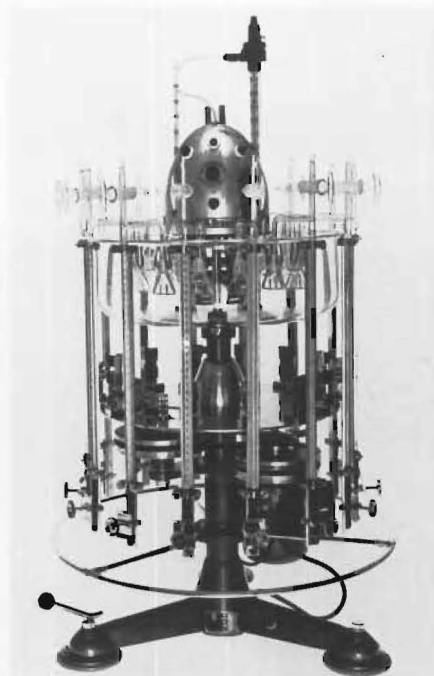
Er. rhusiopathiae was subcultured into 20 replicate tubes containing sterile Medium I, and grown for 24 hours at 37°. The tubes were standardized spectrophotometrically, then centrifuged at 3000 rpm. for 10 minutes in a Wifug, type X1 bench centrifuge. Nine ml of the clear supernatant were removed from each tube and the cells were resuspended in the remaining 1 ml of broth. These suspensions were batched into two tubes, each containing ten 1 ml suspensions. The resulting cultures, which contained approximately 10 times more cells than standardized 24 hour cultures, were used in the manometric experiment. Cultures of D. congolensis incubated at 37° for 7 days in Medium III were treated similarly.

(2) Chemicals:

The water soluble test chemicals were prepared as 6000 µg/ml solutions in sterile distilled water. CCPP, and HCP (TCC was not used in this experiment because of the low activity shown by this chemical in previous tests) were prepared as 6000 µg/ml emulsions using 3% solutions of the emulsifier concentrate. Concentrations of 6000 µg/ml were used so that when 0.25 ml of the chemicals were added to 2.75 ml of culture fluid in the Warburg reaction flasks, the final concentration of chemicals was 500 µg/ml.

(3) Procedure:

The Warburg apparatus model V166 (Fig. 8.1), with reaction flasks number 45 015 (Fig. 8.2) and manometers number 20 205 (Fig. 8.3) (all manufactured by B. Braun, Apparatabau, Melsungen,



**Fig. 8.1 Warburg
Apparatus Model V166**

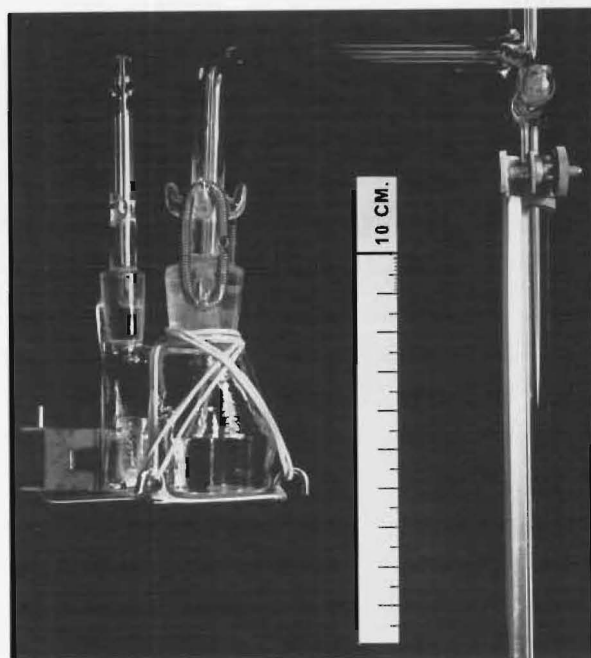
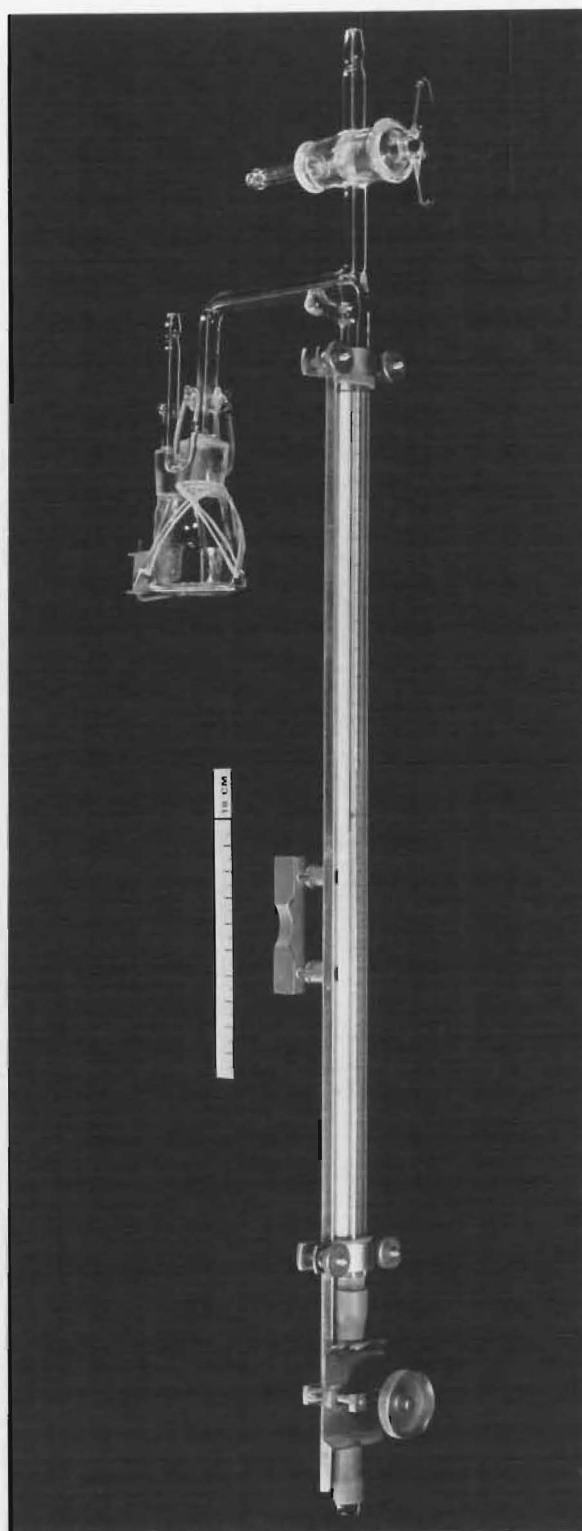


Fig. 8.2 Reaction Flask



**Fig. 8.3 Reaction Flask —
Manometer Unit**

West Germany) was used in this experiment. The temperature of the water bath of this apparatus can be controlled to within 0.01° . In this experiment Brodie's manometer fluid (Umbreit, 1972) made from 23 g sodium chloride and 5 g sodium choleate dissolved in 500 ml water, was used in the manometers.

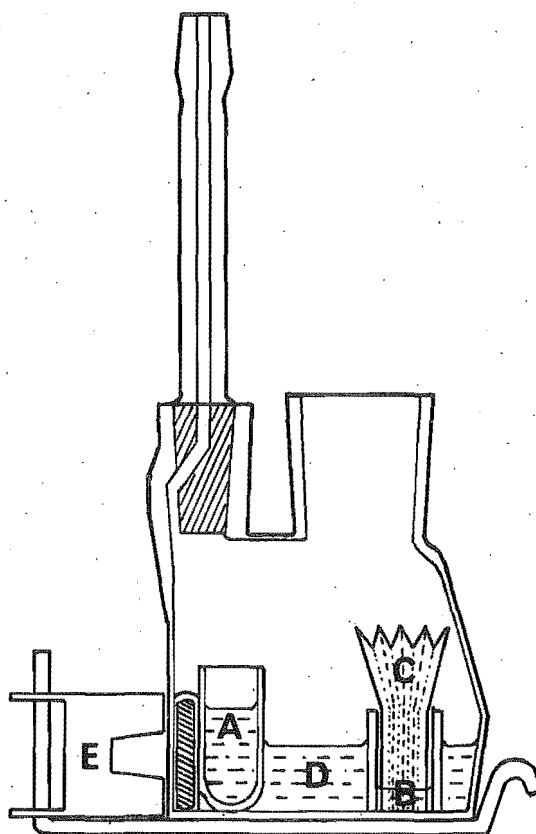
The test procedure was as follows (refer to Fig. 8.4):

The magnetically operated inner vessels (A) were put in place in clean, dry reaction flasks. 0.25 ml quantities of test chemicals were added to the inner vessels using a 1 ml tuberculin syringe. Quantities of 0.2 ml of 10% potassium hydroxide solution were added to the centre wells (B) of each flask using a similar syringe. Alkali absorbed any CO_2 in the flasks so that changes in flask volume were due to O_2 uptake or production by test cultures. The upper rims of the centre wells were lightly greased with silicon jelly using a small glass rod. This was done to prevent KOH from creeping out of the centre well into the test cultures. A 2 cm folded square of Whatman No. 1 filter paper (C) was placed into the KOH in each centre well. Finally, 2.75 ml portions of test culture were placed into each flask (D) using a 2 ml syringe.

For every test run, a thermobarometer was set up to measure changes in atmospheric pressure during the test. This consisted of a reaction flask, identical to those used in the test, containing KOH and a filter paper square in the centre well, but with the chemical and test culture replaced by 3.0 ml of sterile distilled water.

After preparation, the reaction flasks were attached to manometers. The flask-manometer ground glass joints were lightly greased before attachment to prevent gas leaks. The flask-manometer units were then placed onto the Warburg apparatus. The water bath temperature was set at 37.0° and the shaking system was set to give

Fig. 8.4 Diagram of Warburg Reaction Flask
(refer to text)



- A** Magnetically held inner vessel and chemical
- B** KOH in centre well
- C** Folded filterpaper
- D** Culture fluid
- E** Magnet

110 complete 2.5 cm strokes per minute, which, according to Umbreit (1972), should allow adequate oxygen absorption by the culture fluid.

The manometer stop-cock valves were left open for 15 minutes to allow the flask and culture liquid temperatures to equilibrate with that of the water bath. The manometer stop-cock valves were then closed and manometer readings begun. Readings were taken at 10 minute intervals for two hours in the cases of E. coli, E. coli (H₂S), and Ps. aeruginosa, every 15 minutes for 3 hours for Er. rhusiopathiae, and every 30 minutes for 6 hours for D. congolensis. After 65, 98 and 185 minutes respectively, the magnet (M) was removed from each flask, allowing the inner vessel to tip into the culture liquid, thus releasing the test chemical. Readings were continued at the same time intervals until the end of the experiment.

Each test run involved seven reaction flasks. One was used as the thermobarometer; the other six contained test cultures.

II RESULTS

The volume changes that occurred within each flask were recorded as absolute changes in mm of Brodie's solution in manometer tubes. Each flask and manometer were supplied by the manufacturer calibrated for volume. For each flask-manometer unit a flask constant was calculated. By simple multiplication by a flask constant, the observed pressure changes within that flask can be converted directly to a volume of oxygen, in microlitres at standard temperature and pressure (0° and 10,000 mm Brodie's solution), absorbed by the test culture. The conversion was done thus (Umbreit, 1972):

$$\text{Amount of gas exchanged} = \text{change in mm of manometer liquid} \times \text{flask constant (k)}$$

$$\text{Flask constant (k)} = \frac{V_g \times \frac{273}{T} + V_f \alpha}{P_o} :$$

Where V_g = volume of gas phase in flask and manometer,

V_f = volume of fluid in flask

T = temperature of water bath ($^{\circ}\text{K}$)

α = solubility of oxygen in reaction liquid, and

P_o = standard pressure (10,000 mm Brodie's solution)

The manometer readings and the flask constant for the flask-manometer units used have been deposited in the Microbiology Department, Lincoln College. From the converted data, mean oxygen uptake was calculated for each organism and each chemical treatment, and these values were plotted against time. Figs. 8.5, 8.6, 8.7, 8.8 and 8.9 show these plots.

With E. coli, the quaternary ammonium compounds ATMTAC and LDBAC showed the greatest inhibitory effects. HCP, CCPP, arsenic and HPMTS showed some inhibition of this organism. The emulsifier control was slightly inhibitory, but the effect appeared to be short-lived.

E. coli (H_2S) was inhibited sharply by arsenic, ATMTAC and HPMTS. LDBAC was inhibitory while CCPP and HCP showed only slightly more antibacterial activity than did the emulsifier controls.

Ps. aeruginosa was inhibited strongly by LDBAC. ATMTAC, although it took some time (10 to 15 minutes) to show antibacterial effects, was in fact strongly inhibitory also. Arsenic and HPMTS were antibacterial, but to a lesser extent than were the quaternary ammonium compounds. HCP and CCPP were considerably less inhibitory with CCPP showing only slightly greater activity than the emulsifier controls.

Fig. 8.5 Manometrically Determined Effects of Chemicals on Oxygen Uptake by Cultures of *Escherichia coli*. Chemical Concentration 500 μ g/ml

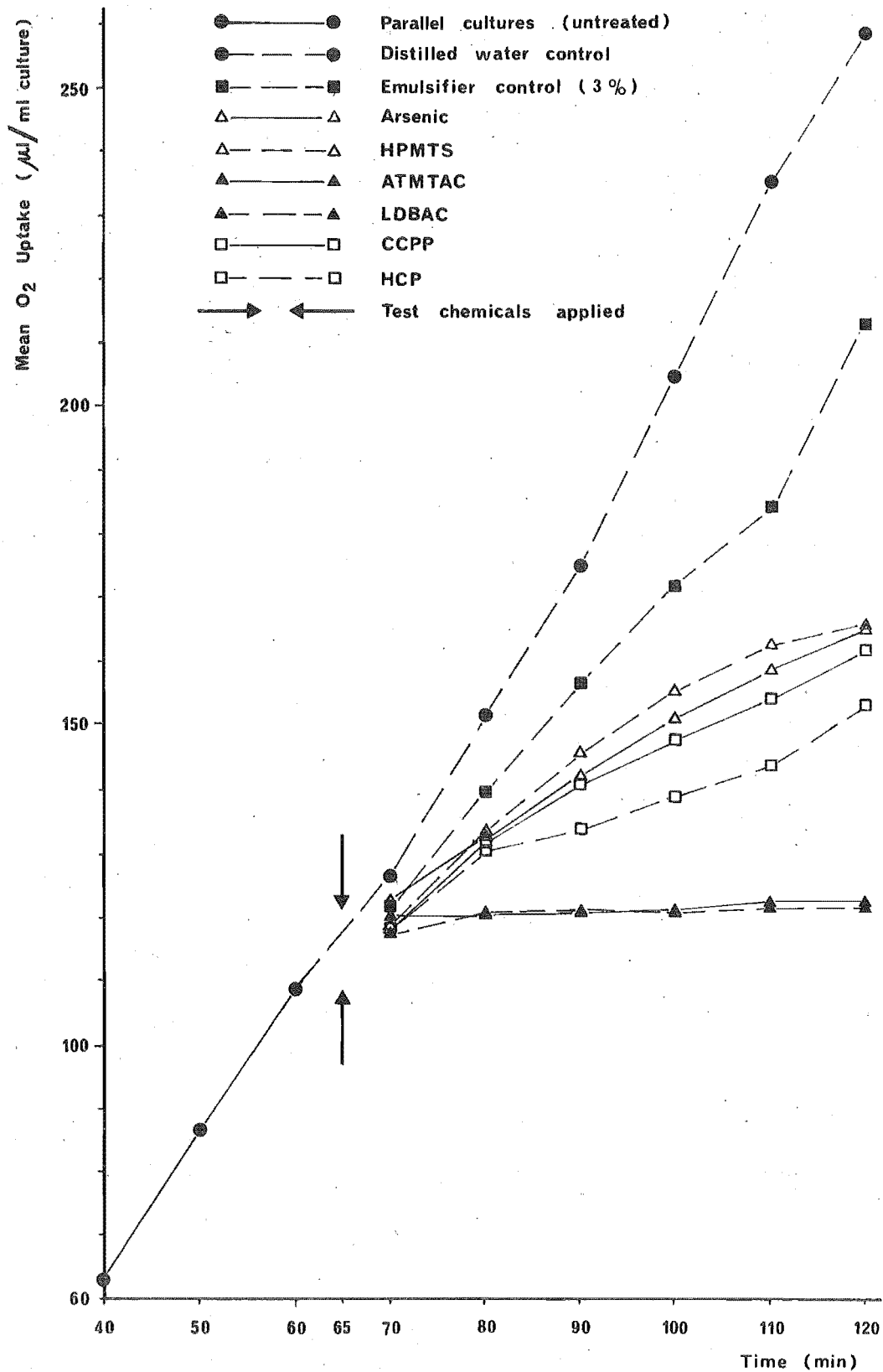


Fig. 8.6 Manometrically Determined Effects of Chemicals on Oxygen Uptake by Cultures of Escherichia coli (H_2S). Chemical Concentration $500 \mu g/ml$

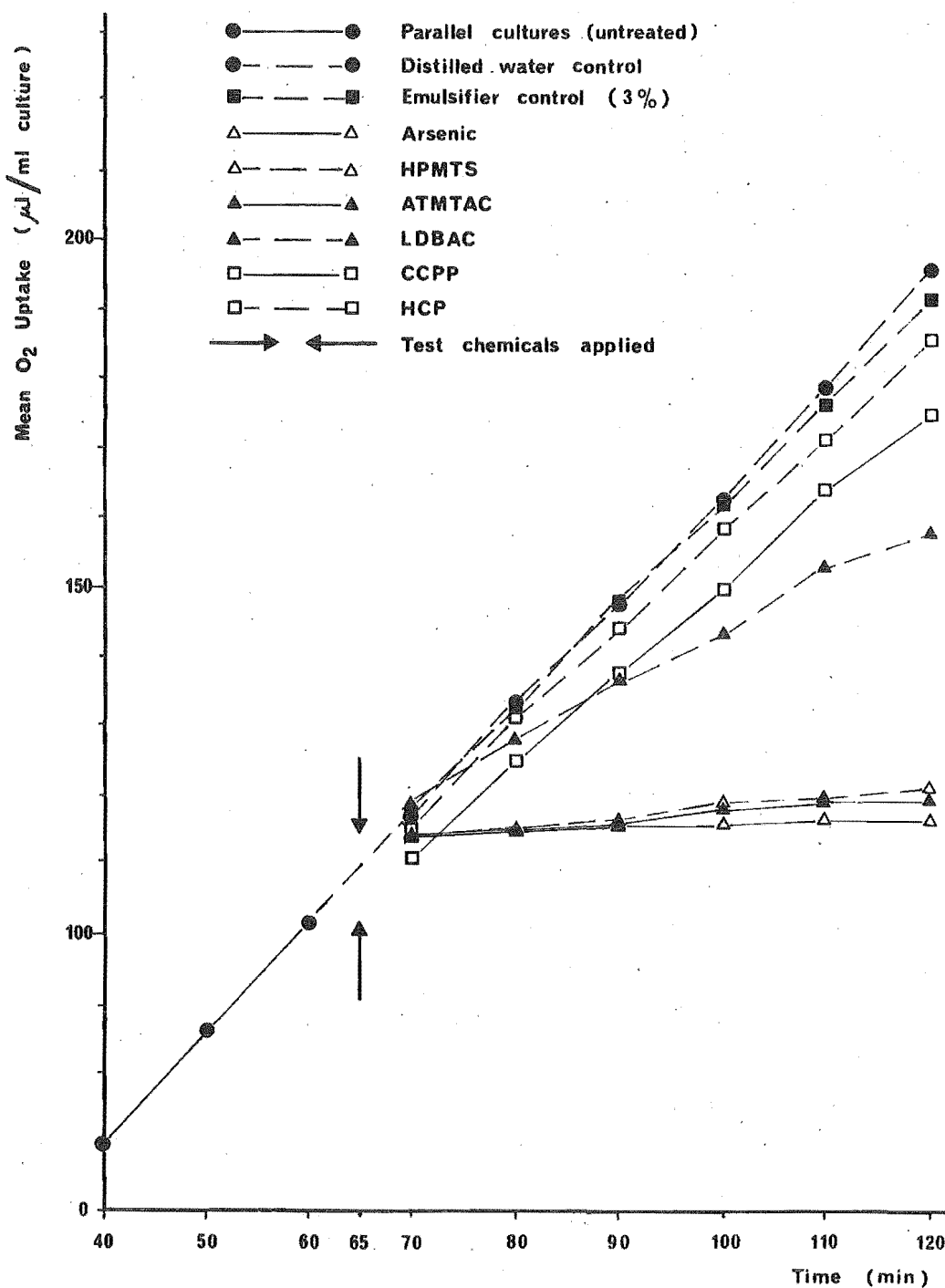


Fig. 8.7 Manometrically Determined Effects of Chemicals on Oxygen Uptake by Cultures of *Pseudomonas aeruginosa*. Chemical Concentration 500 μ g/ml

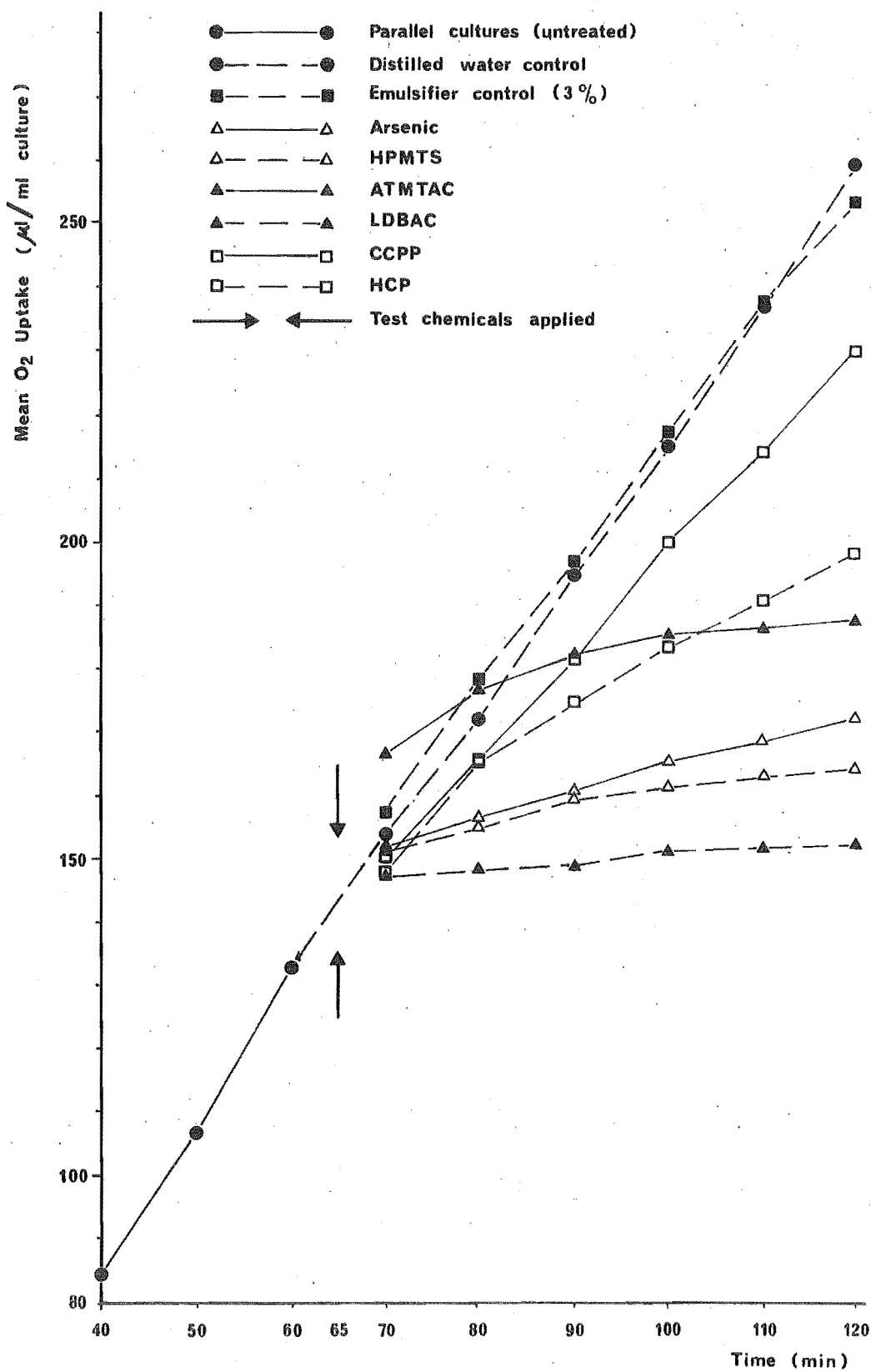


Fig. 8.8 Manometrically Determined Effects of Chemicals on Oxygen Uptake by Cultures of *Erysipelothrix rhusiopathiae*. Chemical Concentration $500 \mu\text{g/ml}$

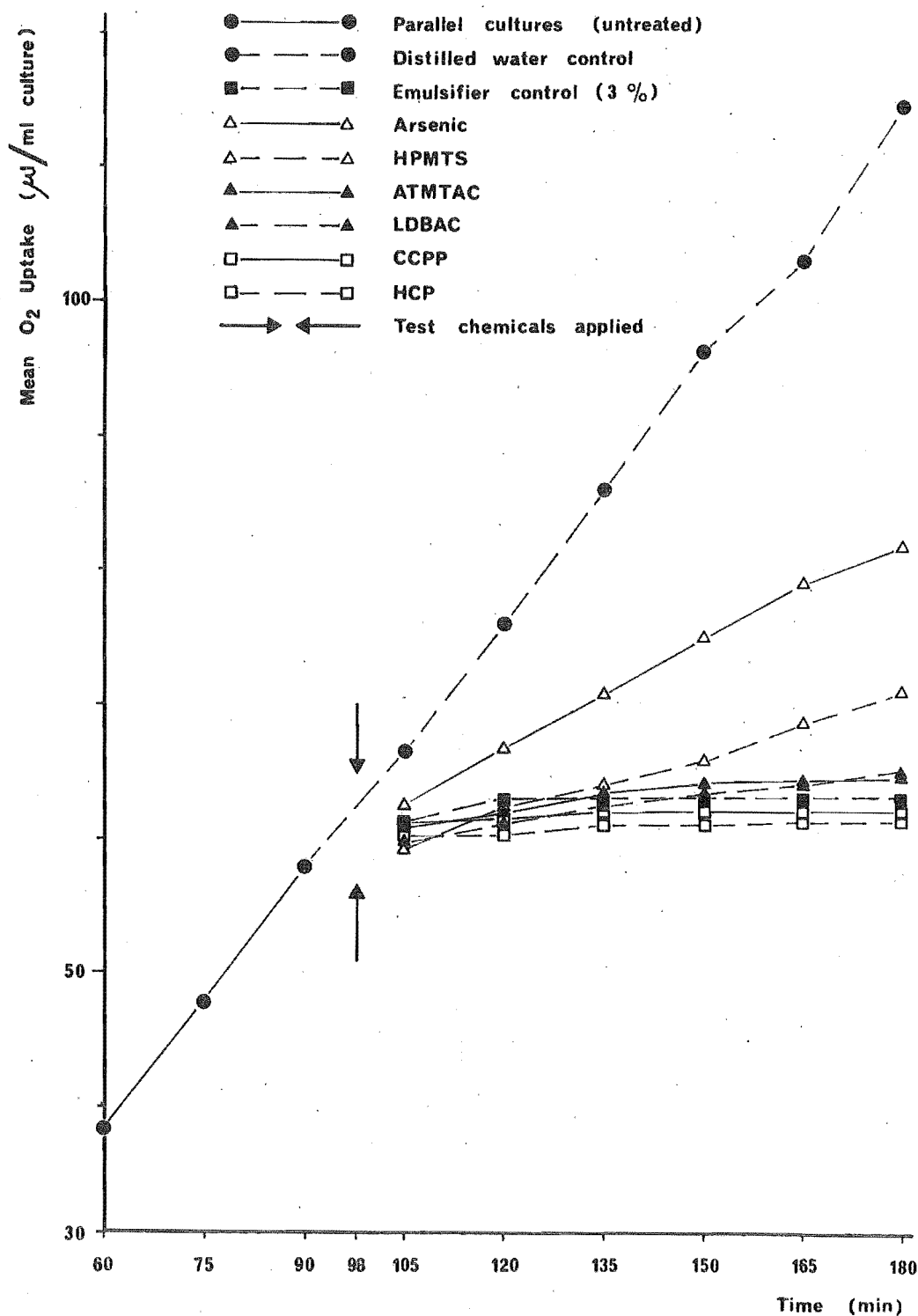
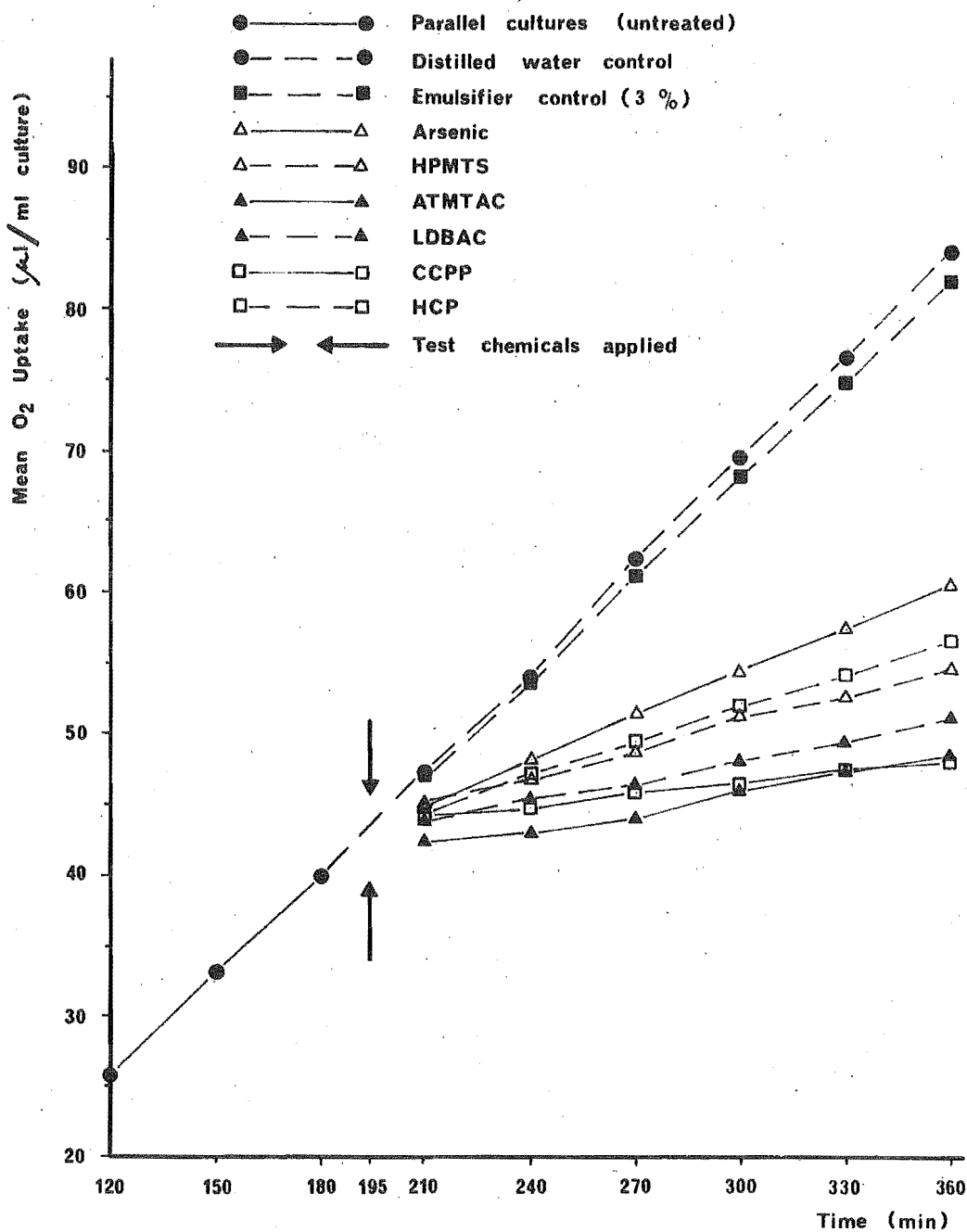


Fig. 8.9 Manometrically Determined Effects of Chemicals on Oxygen Uptake by Cultures of Dermatophilus congolensis. Chemical Concentration $500 \mu\text{g/ml}$



Er. rhusiopathiae was strongly inhibited by the emulsifier, so the inhibitory effects shown by emulsions of CCPP and HCP may be due to this effect. Both of the quaternary ammonium compounds were also strongly inhibitory, while HPMTS and arsenic showed intermediate antibacterial activity.

D. congolensis was inhibited by all the chemicals, with ATMTAC, CCPP and LDBAC being the most effective. HPMTS, HCP, and arsenic were less antibacterial, but all reduced the oxygen uptake of concentrated cultures of D. congolensis to less than half that of the controls.

CHAPTER IX

GRADIENT PLATE METHOD

Szybalski, in a paper by Bryson and Szybalski (1952), briefly described a gradient plate method for isolating strains of bacterial resistant to antibiotics. Szybalski also suggested that the method could be used to measure the sensitivity of bacteria to antibiotics. Since then Szybalski's method has not been widely used, but Bickel et al., (1966) and Sinha (1969) have reported bacterial chemical sensitivities obtained using the method. Sacks (1956) modified the procedure to give a pH gradient plate, and determined the effects of pH upon the anti-bacterial activities of several chemical agents.

The gradient plate method used in this study was based on that described in Bryson and Szybalski's (1952) paper. The procedure created a gradient of gradual proportional increase in concentration of a chemical in agar along one horizontal axis of a Petri plate. The method was simple and required a minimum of equipment. However, gradient plate methods have not as yet been critically evaluated for factors that could cause variability and inaccuracies.

I. MATERIALS AND METHODS

(1) Organisms

Cultures of the test organisms were prepared as described in Chapter V, and were standardized spectrophotometrically where applicable.

(2) Chemicals

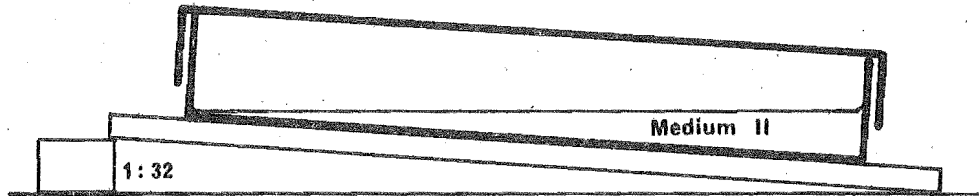
The test chemicals were prepared as 50ml solutions, or emulsions in sterile water at a concentration of 10,000 $\mu\text{g/ml}$. Sterile water and acetone were used as solvents.

(3) Procedure

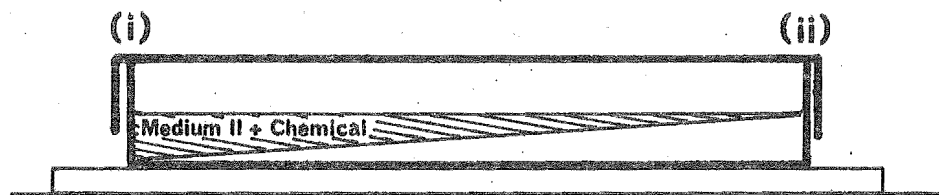
Nine 50 ml portions of double strength Medium II were made up in 200 ml bottles and autoclaved. Also 700 ml of Medium II was prepared and autoclaved. The bottles of Medium II were placed into a water bath set at 50° to keep the medium liquid. The 50 ml solutions and emulsions of test chemicals were added to the 50 ml portions of double strength Medium II so that 100 ml lots of Medium II plus 5000 $\mu\text{g/ml}$ of test chemical were obtained. Sterile water and 3% emulsifier in sterile water were added to double strength Medium II as controls.

Gradient plates were then poured. A previously autoclaved continuous pipetting apparatus (Becton, Dickinson and Company, Rutherford, New Jersey, U.S.A. Catalogue Number 1271) was used to pour 10 ml of plain Medium II into plastic Petri plates inclined on a 1:32 gradient (Fig. 9.1 A). After this layer of agar had gelled, the plates were put onto a flat surface. Using the same continuous pipetting apparatus 10 ml of Medium II-test chemical mixtures were poured into each plate (Fig. 9.1 B). The continuous pipetting apparatus was rinsed with acetone (five deliveries) and sterile water (ten deliveries) between each chemical. The plates were left to gel. The gradient axis was marked on the bottom of each plate. The plates were then inverted, stacked and left on the bench top overnight. Downward diffusion of the chemicals and dilution proportional to the thickness of the two agar layers occurred. In this way a uniform linear concentration gradient was established along one axis of the agar plates. For each test chemical, and for water and emulsifier

Fig. 9.1 Preparation of Gradient Plates
(from Bryson and Szybalski, 1952)



A



B

A 10 ml of Medium II poured with plate on a
1 : 32 inclination

B 10 ml of Medium II plus test chemical
poured with plate horizontal

(i) and (ii) refer to text

controls, six gradient plates were poured, making a total of 54 plates for the test.

The next day the plates were streaked with indicator organisms. On three of the six plates for each chemical, standardized cultures of Escherichia coli (H_2S), and Pseudomonas aeruginosa were streaked along the gradient axis with a wire loop. One loopful of inoculum was used for each streak. A template of cardboard was made to aid streaking of plates. The template had drawn upon it a circle the same size as a Petri plate. Three parallel lines were drawn across this circle, one across the diameter, the other two on either side of the first and 2 cm from it. The plates were orientated on the template so that the concentration gradient corresponded with these lines. The test cultures were streaked along the lines. Plates containing E. coli, E. coli (H_2S), and Ps. aeruginosa were incubated for 24 hours at 37° .

Erysipelothrix rhusiopathiae and Dermatophilus congolensis cultures were streaked onto the three remaining plates containing each chemical. The same template was used, but the streaks were made along the outside two lines and were thus 4 cm apart. These plates were incubated at 37° for 48 hours.

III. RESULTS

After incubation the plates were placed onto another template (Fig. 9.2). On this the diameter of a circle corresponding to that of a Petri plate was divided into eight equal divisions, each of 11 mm. The divisions across the plate could be assigned approximate concentrations as the concentration of test chemical at side (i) (See Fig. 9.B) of each gradient plate was $6000 \mu\text{g/ml}$ and at side (ii) was $0 \mu\text{g/ml}$. The length of the growing streaks across the gradient thus gave a direct measure of the inhibitory concentrations (to the

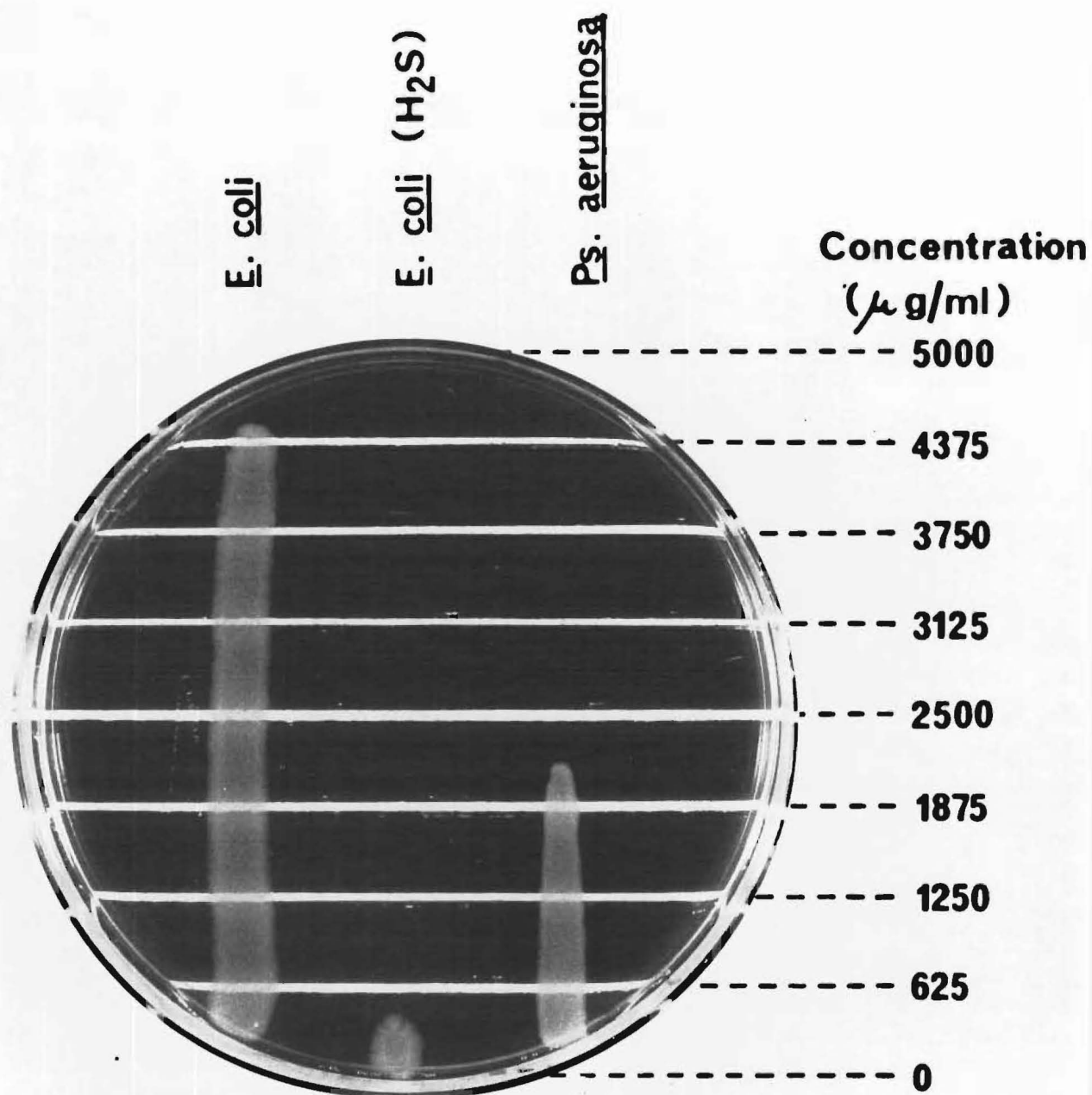


Fig. 9.2 A Gradient Plate on the Concentration Template. Arsenic is the Test Chemical. Plate was Streaked with Organisms Shown and Incubated for 24 h at 37⁰

nearest 625 $\mu\text{g/ml}$) of the seven test chemicals against the five organisms. These approximate inhibitory concentrations are shown in Table 9.1.

E. coli was inhibited most by HPMTS, and slightly less by ATMTAC and LDBAC. Arsenic showed only slight antibacterial activity against this organism, while CCPP, HCP and TCC showed no activity over the concentration range tested.

Against E. coli (H_2S) arsenic was the most effective chemical with HPMTS being only slightly less inhibitory. LDBAC and ATMTAC showed less antibacterial activity against this organism, and CCPP, HCP and TCC did not inhibit it below 5000 $\mu\text{g/ml}$.

Arsenic was the most inhibitory chemical for Ps. aeruginosa. HPMTS, ATMTAC, LDBAC and HCP appeared to show almost identical antibacterial activities, while CCPP and TCC had no inhibitory effects at concentrations below 5000 $\mu\text{g/ml}$.

ATMTAC, LDBAC, and HPMTS were the most effective chemicals against Er. rhusiopathiae with HCP and arsenic being only slightly less inhibitory. CCPP and TCC showed no activity over the concentration range tested.

Against D. congolensis, ATMTAC and LDBAC were the most effective of the chemicals tested, while HPMTS and CCPP showed slightly less activity. HCP and arsenic were reasonably effective, while TCC gave no inhibition of this organism below 5000 $\mu\text{g/ml}$.

Over the range of test organisms, HPMTS was the most effective chemical. ATMTAC and LDBAC were the next most inhibitory chemicals, with arsenic being less antibacterial still. HCP and CCPP were more effective than TCC, which showed no activity against any of the organisms over the concentration range tested.

TABLE 9.1

Results of the gradient plate method.
Minimum inhibitory concentrations ($\mu\text{g/ml}$)
of chemicals against the indicator
organisms, to the nearest 625 $\mu\text{g/ml}$

Organism	<u>E. coli</u>	<u>E. coli</u> (H_2S)	<u>Ps.</u> <u>aeruginosa</u>	<u>Er.</u> <u>rhusiopathiae</u>	<u>D.</u> <u>congolensis</u>
Chemical					
Arsenic	4375 4375 > 5000	625 0 0	1875 1875 2500	625 625 625	1875 1875 2500
HPMTS	0 625 0	0 625 625	2500 3125 3125	0 0 625	0 625 625
ATMTAC	625 625 625	625 1250 1250	3125 3125 3125	0 0 0	0 0 0
LDBAC	625 625 625	1250 625 1250	3125 3125 3125	625 0 0	0 625 0
CCPP	> 5000 > 5000 > 5000	> 5000 > 5000 > 5000	> 5000 > 5000 > 5000	> 5000 > 5000 > 5000	625 625 0
HCP	> 5000 > 5000 > 5000	> 5000 > 5000 > 5000	3125 3125 3125	0 625 625	1250 625 1250
TCC	> 5000 > 5000 > 5000	> 5000 > 5000 > 5000	> 5000 > 5000 > 5000	> 5000 > 5000 > 5000	> 5000 > 5000 > 5000
Water	No Inhibition				
Emulsifier	No Inhibition				

CHAPTER X

FIELD EXPERIMENT

In order to fulfil the first basic requirement of testing of antimicrobial compounds (Bass and Stuart, 1968), data must be interpreted accurately in terms of the practical situations. This may be achieved by translation of in vitro results or by carrying out experiments in vivo. To complete this study it was considered necessary to perform a field experiment to evaluate the results obtained by the in vitro tests.

The experiment carried out was designed to use a single sheep dipping apparatus belonging to the Lincoln College Wool Department. The apparatus was constructed specifically for sheep dipping experiments, and lent itself well to the type of test required by this study.

Two basic approaches were used in the design of the experiment. Firstly, it was considered necessary to determine the effects of the bacteriostatic agents on micro-organisms in the sheep fleece. Secondly, as most of the microbiological problems associated with plunge dipping are related to the dip wash itself, the effects of the test compounds on micro-organisms in this medium would need to be assessed.

Determination of the effects of test compounds on fleece micro-organisms was attempted by inoculating the fleeces of test sheep with cells of Pseudomonas aeruginosa. The numbers

of this organism surviving over a period of time after dipping were studied.

The bacteriostatic properties of test chemicals towards dip wash micro-organisms were determined by effects on the numbers of coliform organisms in the dip washes used in the first part of the experiment. Presumptive coliform counts on dip wash samples were carried out over a period of time after dipping sheep in washes containing the test compounds.

I. MATERIALS AND METHODS

1. Organisms.

Pseudomonas aeruginosa was used as the indicator organism to determine the effects of the test compounds on fleece micro-organisms for several reasons. This organism had been used in all the laboratory tests carried out previously in this study, and had been shown to be the most resistant of the five organisms investigated. It can be easily cultured and may be isolated from mixed microbial populations using selective media. Finally it has been previously shown that this organism is responsible for undesirable fleece stains.

Ps. aeruginosa was subcultured from stock Medium II slopes into 200 ml flasks containing 75 ml of sterile Medium I. The flasks were placed onto a large flask shaker which oscillated once every 9 seconds. The shaker was situated in a warm room controlled at 27°. The cultures were incubated for 48 hours. Plate counts were carried out on samples of these cultures using Modified King's B Medium (described below) and were shown to contain from 1.08×10^{12} to 2.41×10^{12} colony forming units (cfu)/ml.

In order to evaluate the effects of the test compounds on micro-organisms in dip washes after dipping, it was decided to inoculate dip wash samples with Escherichia coli (H₂S) and to determine the numbers surviving over a period of time. Coliform organisms are naturally introduced into dip washes from the dipped animals, but it was not known whether the numbers entering the dip from the small number of animals to be used in the experiment would be sufficient enough to be detected by the usual methods.

Subcultures of E. coli (H₂S) were made from stock Medium II slopes into 10ml portions of Medium I, incubated at 37° for 24 hours and standardized as described in Chapter V.

2. Growth Media.

The enumeration of Ps. aeruginosa in wool samples of test animals was carried out using a plate count procedure and Modified King's B Medium (King et al., 1954; Thom et al., 1971). The medium used was made from 20g/l Bacteriological Peptone (Oxoid Limited, London, S.E. 1, England), 10g/l glycerol (B.P.), 1.5 g/l K₂HPO₄ (May and Baker Limited, Dagenham, England), 1.5g/l MgSO₄·7H₂O (May and Baker Limited), and 15g/l Bacteriological Agar (Davis Gelatine (N.Z.) Limited, Christchurch). This medium was autoclaved at 121° for 20 minutes. Just before plates were poured 25 ml of a 0.2% stock solution of N-(5-nitro-2-furfurylidene)-1-aminohydantoin (nitrofurantoin) in polyethylene glycol 400 (Shell Oil New Zealand Limited) was added to the medium as set out in the paper of Thom et al., (1971).

Presumptive coliform counts on dip wash samples were carried out using MacConkey Broth (Difco Laboratories, Detroit 1, Michigan, U.S.A.) which contained 5g/l oxgall, 20g/l peptone, 10g/l lactose, and 0.01g/l brown cresol purple. The broth was autoclaved in fermentation tubes at 121° for 15 minutes.

The confirming test for faecal coliform organisms chosen required Eosin Methylene Blue Agar (EMBA). The EMBA used in this study was obtained from Oxoid Limited, and contained 10.0g/l peptone, 10.0g/l lactose, 2.0g/l K_2HPO_4 , 0.4g/l eosin Y, 0.06g/l methylene blue and 15.0g/l agar. It was autoclaved at 121° for 15 minutes and poured into Petri plates.

3. Chemicals

Three chemicals were used in this experiment. Arsenic, which had been shown to be the most effective of the test compounds against Ps. aeruginosa in laboratory tests, LDBAC, which was of intermediate activity, and HCP, which was of low activity by comparison, were included. These compounds also showed a range of activity against both of the E. coli strains used in the laboratory tests.

Arsenic and LDBAC were soluble in water. HCP was dissolved in absolute alcohol before adding to water, so that for this chemical a suspension was applied to test animals.

4. Test Animals

Sheep from the Lincoln College Stud Farm Corriedale flock were used. Fifteen ram hoggets that had been shorn 7 weeks previously and had been under cover since the day of shearing were used. The wool staple length was about 2 cm on all the sheep.

Wool characteristics provided by the Wool Department of Lincoln College and relevant to this study are shown in Table 10.1. Sheep with yellow fleeces are considered to be more susceptible to fleece rot (Henderson, pers. com., 1972).

The test animals were held inside throughout the experiment except for a period of about 6 hours on the day of dipping. They were fed each day on a ration of chaff and barley.

5. Sampling of the Wool of Test Animals.

Wool samples of approximately 1g were taken from an area over the rump of test animals using sharp scissors. Samples were taken from across the centre of the back of each animal from an area about 6 cm long by 2 cm wide. The wool was cut from as close to the skin surface as possible, so that about 1 mm of staple was left. Samples were placed into numbered paper bags and returned to the laboratory within 20 minutes of being taken. They were stored for no longer than 3 hours in a refrigerator at 6^o, before they were assayed for Ps. aeruginosa.

6. Inoculation of Fleeces with Indicator Organisms.

Each sheep was prepared for inoculation by spraying the area to be inoculated with a weak solution of detergent. An area on the rump of each animal was sprayed with an 0.2% solution of Mobilsuds (Mobil Oil New Zealand Limited) using a high pressure knapsack sprayer (C.M.W. Industries, Christchurch). Sheep were sprayed twice for 15 seconds each, so that a total of about one litre of detergent solution was sprayed onto an area of approximately 20 by 20 cm of the rump of each animal. The

TABLE 10.1 Some wool characteristics of the sheep used in this experiment.

Sheep Number	Wool Quality	Fleece rot Score	Colour of Fleece
2/71	48	FR wither	3
4/71	58	Clear	2
6/71	54	SFR whole back	3
20/71	56	SFR whole back	2
25/71	54/56	FR wither, loin	4
29/71	48	FR loin	3
34/71	50	Clear	2
38/71	58	FR whole back	3
49/71	58	SFR wither	3
53/71	58	Clear	2
58/71	54	SFR wither	3
74/71	58	Clear	1
78/71	54	SFR whole back	3
79/71	58/60	FR whole back	4
82/71	54	FR wither, loin	3

Fleece rot score SFR = Some fleece rot

FR = Fleece rot

Colour of fleece 1 = 100% white

2 = 25% yellow

3 = 50% yellow

4 = 75% yellow

5 = 100% yellow

applications of detergent solution were at about 10 minute intervals.

After wetting, 40 ml of a 48 hour culture of Ps. aeruginosa were applied to the wool of each of 12 test animals for about 15 cm along the centre of the rump. The other 3 test animals were left uninoculated as controls. The culture was applied from a 100ml measuring cylinder, the mouth of which was used to gently rub the culture liquid into the fleece.

7. Dipping of Sheep.

A single sheep dipping apparatus was used. It consisted of two rectangular tanks, each with a width of 0.71 m, length 1.38 m, and depth 1.02m, mounted on a trailer. The tanks were filled with 900 l of water from a water race, and test chemicals were added as set out below. Sheep were placed singly into a cradle which was attached to the front-end loader of a farm tractor. The cradle was lifted and lowered into the appropriate tank (Fig. 10.1). Each sheep was immersed in the dip wash (Fig. 10.2) for 30 seconds and then lifted out and released.

Five treatments were applied, three sheep receiving each treatment. Sheep were dipped in separate washes containing water only, for the uninoculated sheep, and water only, 600 μ g/ml arsenic, 600 μ g/ml LDBAC, and 600 μ g/ml HCP for inoculated sheep. Except for the three uninoculated sheep, animals were selected randomly for dipping. After each animal had been dipped it was released into a draining pen.

The washes were drained from the dipping tanks when three sheep had been treated. The tanks were scrubbed out and rinsed

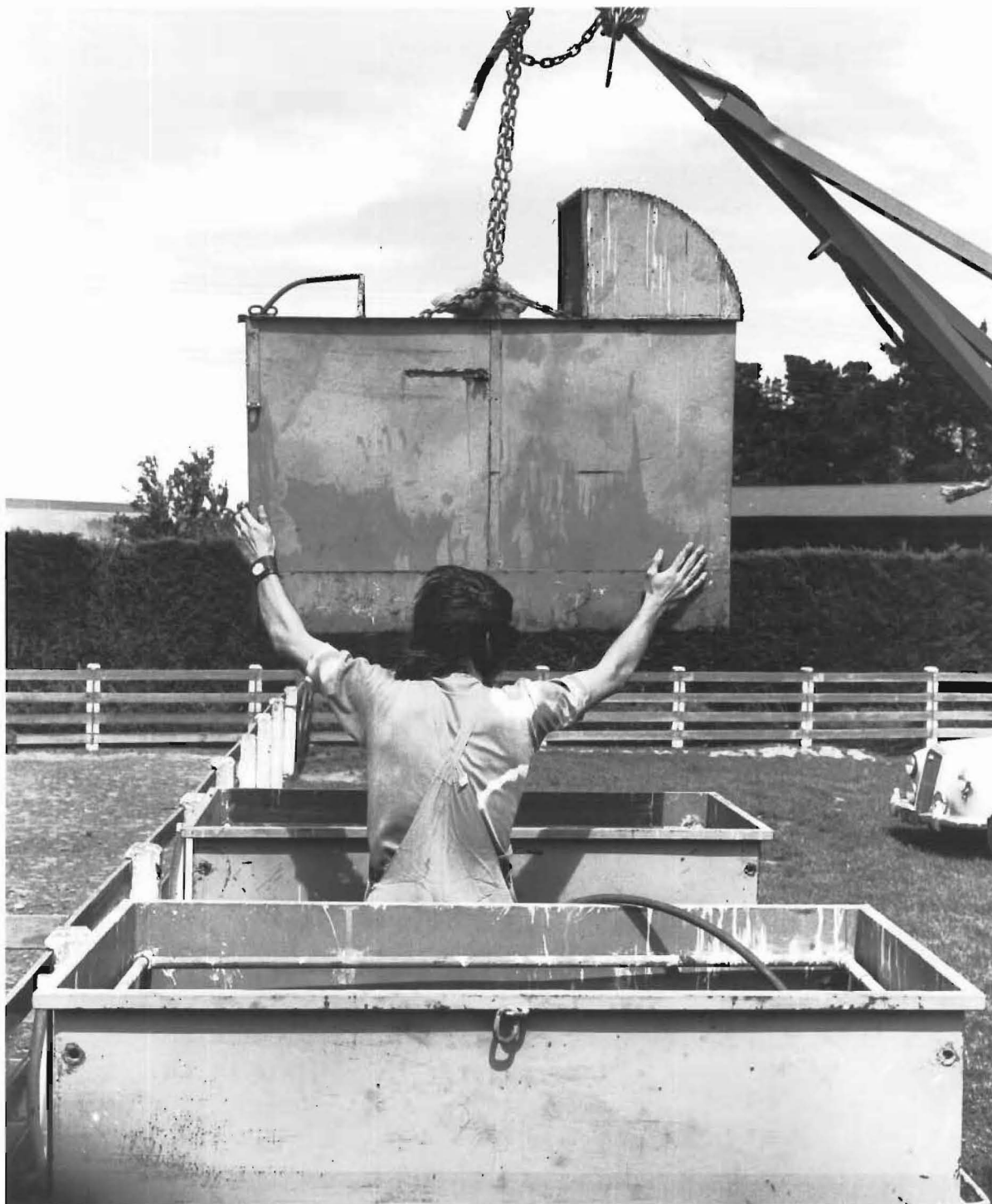


Fig. 10.1 Cradle, containing a test animal, being lowered into one of the dipping tanks

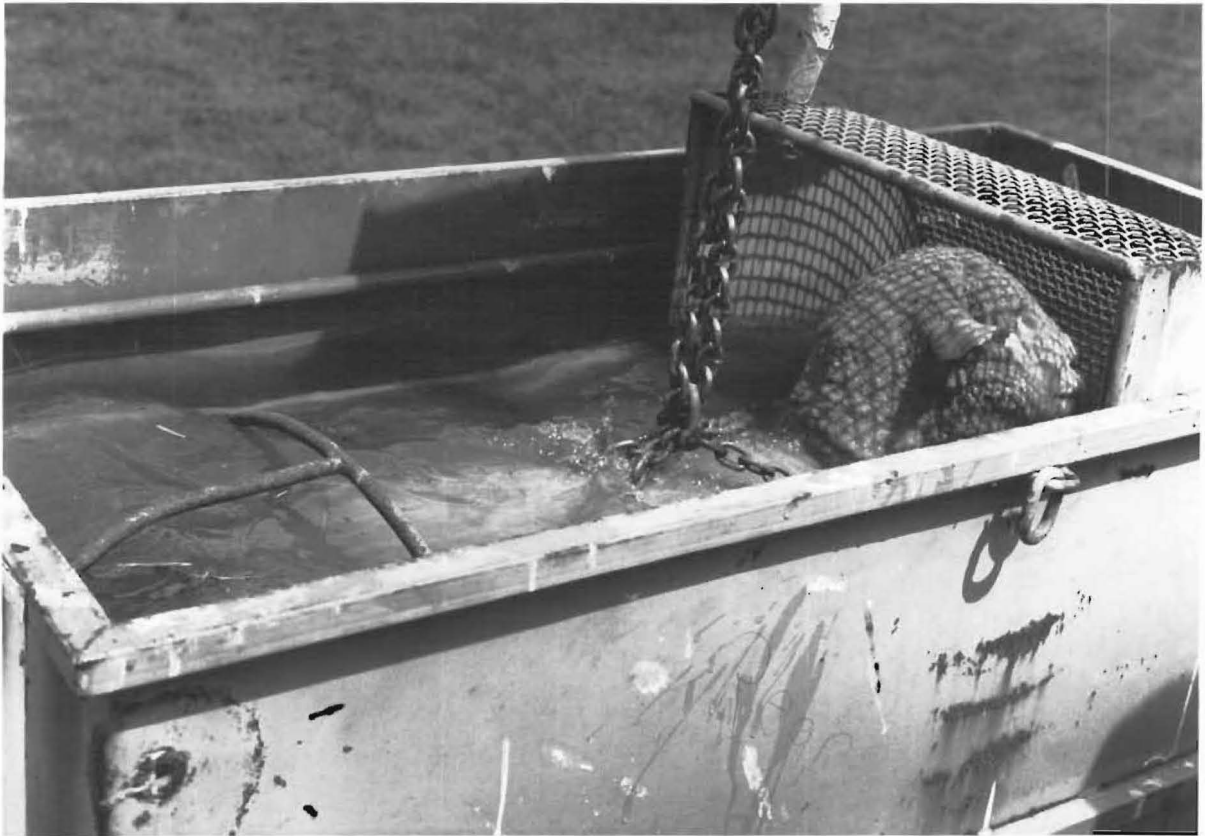


Fig. 10.2 Test animal in dipping tank

with water before being refilled as required.

8. Sampling and Inoculation of Dip Washes.

As the washes were being drained from the dipping tanks, a 2000ml sample was taken from each into a previously autoclaved (121° for 20 minutes) 2 litre flask. The flasks were stoppered with cotton wool bungs and removed to the laboratory. About 2 hours after dipping, each flask was inoculated with 10 ml of a 24 hour standardized (Chapter V) culture of E. coli (H₂S). The flasks were then placed in an incubator at 22°.

9. Enumeration of Ps. aeruginosa in Wool Samples.

A plate count procedure based on that described in Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (1955), p. 373, was used to enumerate Ps. aeruginosa in wool samples. The samples taken from test animals were placed into sterile screw top jars containing 99 ml of sterile water and about 10 glass beads. The samples were shaken in these jars for 1 minute. Serial 10-fold dilutions were made from the solutions in the jars and 1 ml sub-samples of the dilutions were plated in the normal way. Modified King's B Medium was used. The plates were incubated at 37° for 18 to 24 hours. After this time colonies were counted with the aid of a Quebec dark field colony counter (Model 3329, American Optical Corporation, New York, U.S.A.).

Wool samples were removed from the shaker jars, washed, dried and degreased in a Soxhlet apparatus with petroleum ether for 6 to 8 hours. They were then placed in a constant humidity room at 67% R.H. and 22.2°. Samples were weighed after 48 hours in constant humidity conditions.

10. Enumeration and Confirmation of Coliform Organisms in Dip Wash Samples.

Coliform organisms in the dip wash samples held in the laboratory were counted using a most probable number method based on that described in Reports on Public Health and Medical Subjects, Number 71 (1939), p. 21-22.

Four serial 10-fold dilutions of each dip wash sample were made. From each of these, 3 tubes containing 10 ml of double strength MacConkey Broth, 3 tubes containing 10 ml of single strength broth, and 3 more tubes containing 10 ml of single strength broth were inoculated with 10 ml, 1.0 ml and 0.1 ml respectively.

The tubes were incubated at 37° for 24 hours. They were then observed for acid and gas production. All tubes containing both acid and gas were considered presumptive positives.

Presumptive positive tubes from each dip wash sample were tested further by carrying out the confirmed and completed tests for coliforms as described in Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (1955), p. 376-381. Positive tubes were streaked onto EMBA plates and incubated at 37° for 24 hours. Typical Escherichia coli colonies, with a greenish sheen, and dark, almost black centres, were removed from the EMBA plates and reinoculated into MacConkey Broth. These were incubated for 24 hours at 37° and were observed for acid and gas production. Other typical colonies were removed from the EMBA plates, streaked onto Medium II slopes and incubated for 24 hours at 37°. The resulting organisms were Gram stained by the method of Harrigan and McCance (1966).

Presumptive coliform counts were also carried out on the water race water used in the dipping tanks.

11. Experimental Programme.

The following programme was carried out to complete the field experiment:

(a) Two days before dipping (29/10/72)

Wool samples were taken from all the test animals, and assayed for Ps. aeruginosa. The fleeces of 12 of the 15 test sheep were inoculated with Ps. aeruginosa cultures after the wool samples had been taken.

(b) One day before dipping (30/10/72)

Fleeces of all the test animals were sampled and assayed for Ps. aeruginosa. Sheep were transported to the dipping area.

(c) Dipping (31/10/72)

The test sheep were dipped in their respective treatments. Dip wash samples were taken, removed to the laboratory and inoculated with cultures of E. coli (H₂S).

(d) One day after dipping (1/11/72)

Wool samples were taken from test animals and assayed for Ps. aeruginosa. Presumptive coliform counts were carried out on dip samples held in the laboratory.

(e) Two days after dipping (2/11/72)

Wool samples from all test sheep were assayed for Ps. aeruginosa, and dip wash samples were assayed for coliform organisms.

(f) Four days after dipping (4/11/72)

Wool samples and dip wash samples were assayed for Ps. aeruginosa and coliforms respectively. After incubation, confirmed and completed tests for coliforms were carried out on the dip wash samples showing positive presumptive coliforms.

(g) Seven days after dipping (7/11/72)

Dip wash samples were assayed for presumptive coliforms. Confirmed and completed tests were carried out on tubes from dip wash samples showing presumptive coliforms.

(h) Ten days after dipping (10/11/72)

Dip wash samples were assayed a final time for coliform organisms using presumptive, confirmed and completed tests as before.

II. RESULTS

1. Numbers of Ps. aeruginosa on wool samples.

Thom et al., (1971) considered that the Modified King's B Medium they described was suitable for assessing numbers of Ps. aeruginosa in human faecal specimens by direct plating. All the colonies from wool samples assayed in this study were considered to be Ps. aeruginosa colonies, and numbers in wool samples are reported from colony counts on this basis. All counts were converted to cfu/g clean wool conditioned to constant weight at 67% R.H., 22.2°. These values are shown in Table 10.2. Wool weights are recorded in Appendix 3, Table A3.1.

TABLE 10.2

Results of plate counts on wool samples. Numbers of colony forming units/g clean wool conditioned to constant weight at 67% R.H. and 22.2°, as indicated on Modified King's B Medium (Thom et al., 1971).

Sheep Number	Treatment	Date				
		29/10/72	30/10/72	1/11/72	2/11/72	4/11/72
2/71	Water Uninoc'd	6.07×10^4	1.08×10^5	$< 10^4$	$< 10^4$	8.30×10^5
34/72		1.01×10^5	2.77×10^5	$< 10^4$	8.79×10^4	9.70×10^4
38/71		1.99×10^5	5.91×10^5	$< 10^4$	$< 10^4$	4.44×10^5
20/71	Water Inoc'd	5.87×10^4	3.66×10^{11}	$< 10^4$	2.73×10^5	3.30×10^6
25/71		1.98×10^5	4.34×10^{11}	$< 10^4$	3.70×10^5	6.32×10^5
53/71		1.08×10^5	1.51×10^{11}	$< 10^4$	$< 10^4$	2.10×10^5
49/71	Arsenic 600 μ g/ml Inoc'd	1.57×10^5	1.88×10^{10}	$< 10^4$	1.62×10^5	3.56×10^5
58/71		1.29×10^5	3.58×10^{11}	$< 10^4$	8.48×10^4	1.49×10^5
79/71		7.54×10^4	7.66×10^{10}	$< 10^4$	$< 10^4$	2.96×10^5
4/71	LDBAC 600 μ g/ml Inoc'd	1.13×10^5	9.97×10^{10}	$< 10^4$	1.35×10^5	7.79×10^4
29/71		7.73×10^4	2.21×10^{12}	$< 10^4$	9.57×10^4	3.28×10^4
78/71		4.17×10^5	1.10×10^{10}	$< 10^4$	$< 10^4$	1.29×10^5
6/71	HCP 600 μ g/ml Inoc'd	1.84×10^5	1.97×10^{11}	$< 10^4$	$< 10^4$	5.41×10^5
74/71		2.59×10^5	1.14×10^{12}	$< 10^4$	$< 10^4$	2.03×10^5
82/71		4.30×10^5	1.87×10^{10}	$< 10^4$	2.98×10^5	2.04×10^5

Test animals inoculated with Ps. aeruginosa just after wool samples were taken on 29/10/72.

Sheep dipped in treatments indicated on 31/10/72.

Natural populations of Ps. aeruginosa occurred in the fleeces of the test sheep. These varied from between 10^4 to 10^5 cfu/g wool. Inoculation of the test animals was successful and yielded from 10^{10} to 10^{12} cfu/g wool when assays were carried out 1 day after inoculation.

Dipping and the handling of the animals that this involved reduced the numbers of pseudomonads in the wool of all test sheep to below the level that could be detected by the plate counting method used. Two days after dipping the natural levels of Ps. aeruginosa reappeared in the wool of some of the sheep, and by 4 days after dipping all sheep had normal populations of this organism. No effects of the bacteriostatic agents could be detected.

It is speculated that exposure of the natural populations of Ps. aeruginosa in the fleeces of test animals to direct sunlight on the day before dipping may have brought about the considerable decrease in numbers that occurred. The short wool of the test sheep would have afforded little protection from U.V. sunlight. However, the reasons for the drop in numbers are most probably far more complex than this, and the dipping process itself could well be involved.

2. Presumptive Counts of Coliform Organisms in Dip Washes.

The presumptive numbers of coliform organisms in the dip wash samples were determined from the numbers of MacConkey Broth tubes showing positive acid and gas production. Most probable number (MPN) tables from International Standards for Drinking

Water (1963) were used for this purpose. MPNs of coliforms with upper and lower 95% confidence limits are shown in Appendix 3, Table A3.2. Fig 10.3 shows the plots of the numbers of presumptive coliforms in dip washes from the five treatments used. No coliforms were detected in the water race water used in the experiment.

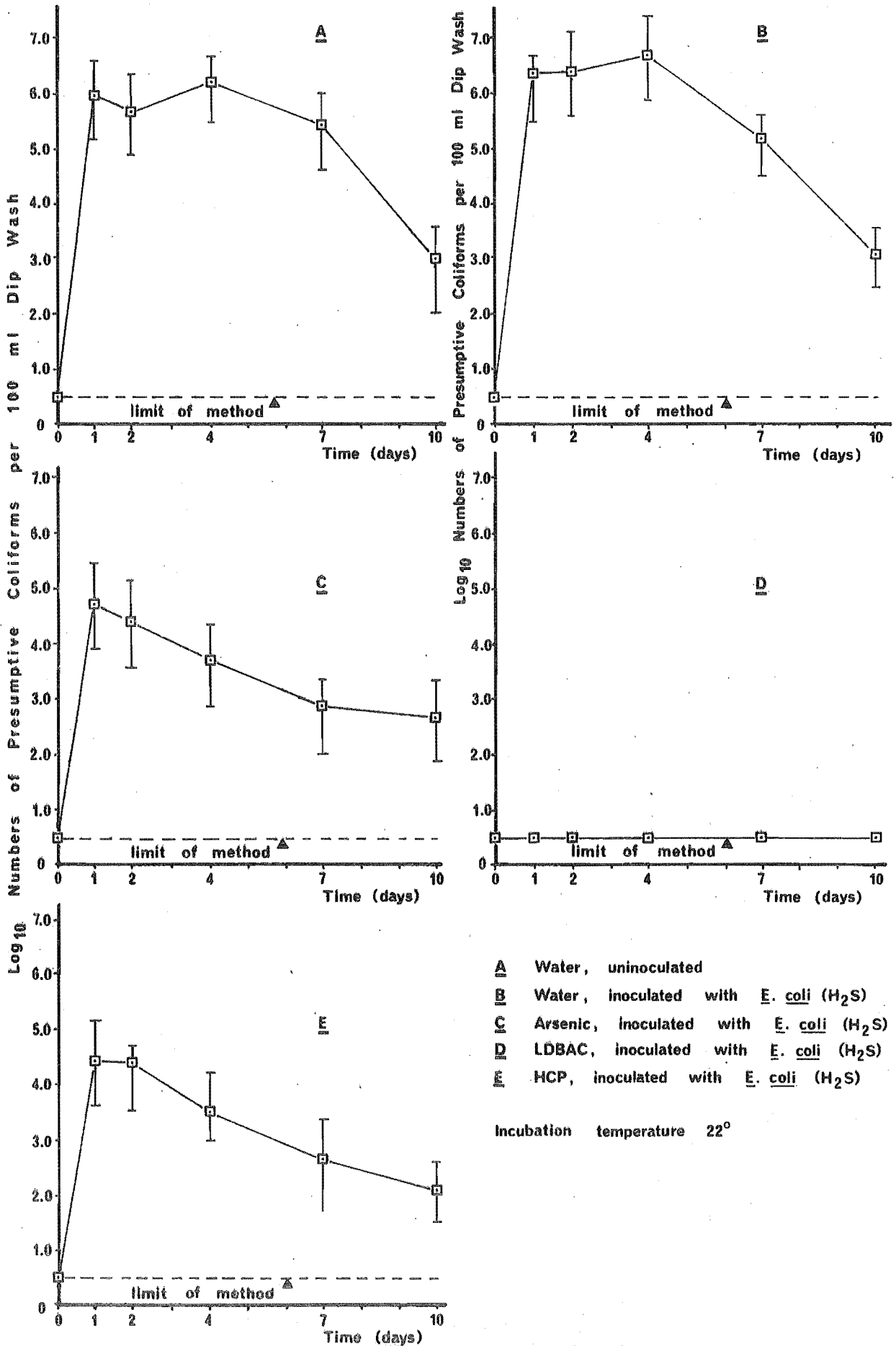
Both uninoculated and inoculated dip wash samples containing water only (Fig. 10.3, A and B) showed similar numbers of presumptive coliforms. Inoculation of water with E. coli (H_2S) made no significant difference (within 95% confidence limits) to the numbers of coliforms present.

Arsenic and HCP had comparable effects on coliform numbers. Both compounds prevented multiplication to some extent, and the rate of decrease in numbers appeared to be more rapid than that occurring in the water controls. The differences between arsenic and HCP were insignificant within 95% confidence limits.

Of the chemicals tested, only LDBAC prevented multiplication of coliforms introduced into the dip wash at the time of dipping. At no stage up until 10 days after dipping were coliforms detected in the wash containing 600 $\mu\text{g/ml}$ of this quaternary ammonium compound.

All the dip wash samples held in the laboratory except that containing LDBAC darkened to some extent, indicating the metabolic activities of sulphur reducing bacteria. However, no method other than visual inspection was used to determine the degree of blackening that occurred.

Fig. 10.3 Presumptive coliform counts of dip washes after dipping three sheep in each. Upper and lower 95% confidence limits indicated



All the dip wash samples showing positive presumptive coliforms, namely both control washes and those containing arsenic and HCP, gave completed tests for coliform organisms. Typical colonies were obtained on EMBA plates, and gas was produced by organisms from these colonies when they were inoculated into MacConkey Broth. Gram stained smears showed Gram-negative, non-sporing bacilli from 3.1 to 3.6 μm long by 0.5 μm wide.

SECTION 3

DISCUSSION AND CONCLUSIONS

CHAPTER XI

THE EFFICACY OF THE CHEMICALS TESTED AS
BACTERIOSTATIC AGENTS FOR USE IN SHEEP
DIPS

The major purpose of this study has been to assess the effectiveness of six chemical compounds as bacteriostatic agents for use in sheep dips. For this reason, the efficacy of the chemicals as sheep dip bacteriostats is considered first.

Two criteria have been used in the consideration of each compound. Firstly, the effectiveness in vitro of each chemical in preventing the growth of the indicator organisms investigated was assessed. As the indicator organisms had been chosen for their ability to cause problems associated with plunge dipping, and undesirable conditions of fleece wool, the prevention of their growth would give some indication of the value of the test chemicals in a practical situation.

Secondly, as far as could be established, the toxicity of the test compounds to both animals and humans must be taken into consideration. One of the reasons why arsenical bacteriostats are undesirable is because of their high mammalian toxicities. A bacteriostat to replace arsenicals should not have the same undesirable characteristics.

Each of the test compounds is considered separately in the light of these criteria.

I. THE TEST COMPOUNDS

1. 2-Hydroxypropylmethanethiosulphonate (HPMTS)

HPMTS was the most effective of the test chemicals against Escherichia coli, E. coli (H_2S) and Pseudomonas aeruginosa, the three Gram-negative organisms tested. This compound was only moderately active against Erysipelothrix rhusiopathiae and Dermatophilus congolensis.

HPMTS has been approved as a slimicide for use in the manufacture of food contact paper and paperboard in the U.S.A. (Anon., 1970). No other reference has been found in the literature to this compound. On this information it can be assumed that HPMTS is relatively non-toxic. More information should be obtained, however, before it is included in sheep dip preparations.

2. The Quaternary Ammonium Compounds

Lauryldimethylbenzylammonium chloride (LDBAC) and the mixture of alkyl-, tolyl-, and methyl- trimethylammonium chlorides (ATMTAC) showed very similar antibacterial activities against all the indicator organisms. Also these compounds showed uniformly high activity against all the organisms.

Lawrence (1968) listed limiting dilutions of some alkyldimethylbenzylammonium chlorides, and indicated that these compounds were more active against Gram-negative than Gram-positive organisms. However, the results of this study showed very little

difference in susceptibility between the two groups. The modes of inhibitory action suggested for these compounds against bacterial cells (Lawrence, 1968) and varied. Quaternary ammonium compounds appear to be responsible for interfering with respiration and glycolysis, to cause disruptions to membrane permeability, and to be able to split lipoprotein complexes, thus allowing autolytic enzymes to become active and bring about cell lysis. The fact that these chemicals have several sites of action may account for their apparent broad antibacterial spectrum.

Quaternary ammonium compounds appear to be relatively non-toxic to both humans and animals. It has been found that 0.3% of a quaternary ammonium compound in the diet of animals caused no ill effects (Lawrence, 1968). LDBAC was shown to be an effective bacteriostat for coliform organisms at 600 $\mu\text{g/ml}$ in the field experiment carried out in this investigation, a concentration well below the levels shown to cause toxicity symptoms in animals (Lawrence, 1968).

3. 4-Chloro-2-cyclopentylphenol (CCPP)

CCPP showed generally low antibacterial activity against all the test organisms except D. congolensis, against which it was highly active. It appeared to be highly active against E. coli in the broth dilution test and the agar diffusion test, but the manometric and gradient plate tests indicated that the activity against this organism was low.

Little could be established regarding the toxicity of this compound although Prindle and Wright (1968) pointed out that an increase in the molecular weight of phenol derivatives may be accompanied by decreasing animal toxicity.

4. 2,2'-Dihydroxy-3,5,6,3',5',6'-hexachlorodiphenylmethane (HCP)

HCP was relatively uninhibitory to the three Gram-negative organisms tested, namely E. coli, E. coli (H₂S), and Ps. aeruginosa. This result is in agreement with the findings of other authors (Gump and Walter, 1968; Kneiflova and Privora, 1970). Hugo and Bloomfield (1971a, b and c) suggested that E. coli was more resistant than Staphylococcus aureus to the bis-phenolic compound 2,2'-dihydroxy-5,5'-dichlorodiphenylsulfide due to the lipid rich nature of the cell wall of E. coli. The cell wall of Gram-negative bacteria may adsorb bis-phenolic compounds preventing them from reaching the site of action, which appeared to be the cell membrane systems. HCP showed reasonable activity against the Gram-positive organisms Er. rhusiopathiae and D. congolensis.

The field experiment showed that HCP has some inhibitory effects on coliform organisms in dip washes. The higher activity of this chemical against Gram-positive organisms may suggest that HCP would give good control of post-dipping lameness and ovine cutaneous actinomycosis.

Recently there has been considerable publicity in the popular press on the mammalian toxicity of HCP. The scientific literature has also contained references to the problem (Kimbrough, 1971). HCP has been widely used as a human skin antibacterial agent, and applications of high concentrations to infants in particular seem to be unwise. However, as the central nervous system toxic signs and symptoms appear to be short-lived when treatment with the compound is discontinued (Kimbrough and Gates, 1971), HCP toxicity should not be a

problem in sheep dipping.

5. 3,4,4'-Trichlorocarbanilide (TCC) .

By comparison with the other test compounds, TCC showed only very limited activity against all the indicator organisms used in this study. In fact, only in the agar diffusion test was any activity detected at the concentrations tested, and in this case only D. congolensis was inhibited by the compound. From these results it would appear that TCC has little or no value as a sheep dip bacteriostatic agent.

6. Arsenic

Sodium arsenite remains one of the more effective compounds for the control of bacteria associated with sheep dipping. In this study arsenic was the most effective of the chemicals tested against E. coli (H_2S) and Ps. aeruginosa, and showed moderate activity against Er. rhusiopathiae and D. congolensis. However, as mentioned previously, the occurrence of arsenical scald in sheep dipped in washes containing arsenic, and the high mammalian toxicity of arsenical compounds, makes them undesirable for sheep dipping.

II. CONCLUSIONS

All of the compounds tested in this study except TCC have some value as sheep dip bacteriostatic agents. The quaternary ammonium compounds appear to be the most satisfactory because of their broad spectrum of antibacterial activity, and their low toxicity at concentrations required to control bacterial growth. It is suggested that the addition of a quaternary ammonium compound to plunge dipping baths could be

useful for controlling post-dipping lameness and dip wash blackening, and to control undesirable fleece micro-organisms, the most important of which appears to be D. congolensis.

The other test compounds have more limited value as dip wash bacteriostatic agents. HPMTS was particularly effective against the Gram-negative organisms, but was less effective against Er. rhusiopathiae and D. congolensis. CCPP was the most effective antibacterial compound against D. congolensis. HCP was of limited activity against the Gram-negative organisms, but could be of use for the control of post-dipping lameness and ovine cutaneous actinomycosis.

It is possible that combinations of bacteriostatic agents may be of value for the control of bacterial organisms in dip washes and fleece wool. In this regard further testing is required. Some combinations of bacteriostatic agents bring about antagonistic effects between compounds, and a consequent reduction in antibacterial activity. HCP and quaternary ammonium compounds have been shown to produce these effects (Gump and Walter, 1968).

CHAPTER XII

COMPARISON AND DISCUSSION OF THE
METHODS USED TO ASSESS THE ANTIBACTERIAL
ACTIVITY OF TEST COMPOUNDS

Five methods were used in this investigation to evaluate the test compounds as sheep dip bacteriostatic agents. In using the techniques outlined it has become obvious that several points should be made concerning sensitivity tests in general and with the testing of compounds for the specific use considered in this study.

The five methods used were a broth dilution turbidimetric and agar subculture method, an agar diffusion method, a manometric technique, a gradient plate method, and a field experiment. The procedures will be discussed separately, and some suggestions made concerning standard tests for sheep dip bacteriostatic agents.

I. DISCUSSION OF THE METHODS USED IN THIS STUDY

1. Broth Dilution Turbidimetric and Agar Subculture Method.

Dilution methods of sensitivity testing are the most commonly used in vitro procedures to assess the antibacterial activity of chemical agents. It was for this reason that the broth dilution method was adopted.

The procedure involved a considerable amount of manipulation of simple laboratory equipment. Dilution series and replicates required large numbers of sterile tubes containing sterile broth medium. The addition of chemicals to tubes was a tedious operation, and each tube had to be inoculated separately with indicator organisms. Thus the setting up of the first part of the test was a time-consuming procedure.

Reading of broth dilution results is simple and rapid, but some difficulties may arise with end-point determinations. This problem was only slight in this study, possibly because of the rather wide dilution steps used.

The second part of the test also required a considerable amount of rather tedious work, as each tube from the dilution series had to be again handled when agar plates were streaked. It is possible that automated micro-dilution techniques, as suggested by Chitwood (1969), would remove much of the tedium from this type of method.

Broth dilution turbidimetric methods are simple but tedious. The addition of agar subculturing to the procedure increases the technical work load considerably, but provides additional valuable information on the activity of test chemicals.

2. Agar Diffusion Method

The agar diffusion method used required simple laboratory equipment. Some care was required in the choice of Petri plates, as uniformity in these was essential. The procedure required a total of 45 Petri plates to test 7 chemicals and controls against 5 indicator organisms. Pouring and setting of and application of chemicals to these could be achieved in less

than 3 hours. Results could be obtained in 24 hours for faster growing organisms, but longer for organisms such as Erysipelothrix rhusiopathiae and Dermatophilus congolensis.

Perhaps the most valuable feature of the method used was the particularly low coefficients of variation in the results obtained (Appendix 2, Tables A2.4, A2.5, A2.6, A2.7, and A2.8). These result from the considerable effort that has been made to consider and overcome the factors causing variability and error in agar diffusion procedures of this type (Davis and Stout, 1971a).

Another feature of the method was its sensitivity. Significant differences in the antibacterial activities of test compounds could be detected even when mean inhibition zone diameters differed by only small amounts. This was the only method of the three in which TCC was tested that detected antibacterial activity by this compound. The procedure allowed quantitative comparison between compounds of dissimilar levels of activity.

The agar diffusion method gave some indication of an area of knowledge where some investigation has been carried out but where a paucity of information exists. Hormetic responses by micro-organisms to chemicals would be a fruitful area of study particularly for biochemical microbiologists.

3. Manometric Method.

The manometric method used in this study employed the Warburg apparatus of the Microbiology Department at Lincoln College. This equipment is specialized and expensive. The reaction flasks used in this test (Fig. 8.2) are delicate

and expensive pieces of glassware, but were essential for the accurate addition of small volumes of test chemicals to cultures without interfering with the test. Thus the major drawback of the method was the expense of the equipment involved.

The method was rapid, with the longest duration of a test run being 6 hours for D. congolensis. However, if only one Warburg apparatus is available the numbers of chemicals and organisms that can be tested at one time is limited. The whole test took 4 weeks to complete. This would have been reduced by 2 weeks if twice as many flasks of the type used had been available, so total time taken could have been shortened.

The manometric technique used was very sensitive. It was relatively simple to detect small differences in the antibacterial activities of the test compounds. High sensitivity was a disadvantage in one case however. This was the only method that detected any antibacterial activity in the emulsifier concentrate used throughout this study and this masked the activities of emulsions that were tested.

4. Gradient Plate Method

The gradient plate test used was relatively insensitive to differences in antibacterial activities of chemicals. The advantages of this method are simplicity and speed with which it can be performed. For these reasons the method may be of value in preliminary screening studies. However, it is the opinion of the author that only those chemicals which are active at very low concentrations can be accurately tested with this method. Antibiotics, the study of which was the purpose of the originally described gradient plate procedure (Bryson and

Szybalski, 1952), may lend themselves more readily to this type of test.

5. The Field Experiment

The area where the greatest need for investigational work on the microbiology of sheep dipping concerns the sheep dip itself. The emphasis in this study was on laboratory testing, and the field experiment was a relatively minor part of the research project. Nevertheless, it was the field experiment that indicated the need for future investigations of a more practical nature.

Very little is understood about the interactions between microbial populations living in sheep dip washes. The environment contains adequate water, organic and inorganic nutrients, and is at a temperature that allows micro-organisms to multiply. Oxygen conditions may become limiting to the growth of strict aerobes. That the environment provided by a dip wash in which numbers of sheep have been dipped is suitable for the growth of bacteria of practical importance is obvious from the fact that populations of Er. rhusiopathiae and Escherichia coli have been shown to multiply very rapidly in it.

The dipping experiment gave positive results only in the section concerned with dip wash micro-organisms. The effects of bacteriostatic agents on micro-organisms in the fleece have not been established. Conclusions drawn about the practical effectiveness of the test compounds against Pseudomonas aeruginosa, Er. rhusiopathiae and D. congolensis are based solely on in vitro results and must therefore be confirmed in the field.

It was relatively simple to obtain controlled experimental conditions with the type of field procedure used in this investigation. Further consideration of practical sheep dip microbiology would be of both theoretical and practical value.

II. LACK OF CORRELATION BETWEEN RESULTS OBTAINED FROM THE TEST METHODS USED.

The results obtained with E. coli were the most inconsistent of those for all the indicator organisms tested. Two examples are obvious. CCPP was shown to be the most inhibitory of the chemicals for this organism by both the broth dilution turbidimetric and the agar diffusion methods, but was one of the least antibacterial compounds in the manometric and gradient plate tests. LDBAC and to a lesser extent ATMTAC showed almost the reverse tendency, being relatively less effective against E. coli in broth dilution and agar diffusion tests but more effective as indicated by the manometric and gradient plate methods.

These inconsistencies cannot be explained. However, as it is well known that the antibacterial activities of chemicals may be markedly affected by physical chemical factors (Kostenbauder, 1968), the lack of correlation that occurs in some cases may be due to complex interactions between chemicals and the micro-environment of indicator micro-organisms. This lends even more weight to the suggestion of Heatley (1949b) that all the experimental conditions under which a sensitivity test is performed should be set out fully.

III. SUGGESTIONS FOR A STANDARD TEST FOR SHEEP DIP BACTERIOSTATIC AGENTS

A line of investigation that was not followed in this study was the development of a laboratory test that attempted to simulate the conditions of the practical situation under consideration. Experiments have been carried out to simulate field conditions with some success (Wright and Pelham, 1968). It is possible that growth media containing the organic nutrient materials found in dip washes could be used to evaluate the effects of chemicals on indicator organisms.

On the other hand, it is apparent that there are in existence methods that provide all the requirements of good standard tests for sheep dip bacteriostatic agents. The agar diffusion method used in this study, which closely follows that described by Davis and Stout (1971b), gave a sensitive and statistically acceptable test with a minimum of equipment. The degree of correlation between the results obtained with this method and the information provided by the field experiment was good. It thus seems reasonable to recommend the adoption of this test procedure as a standard method for testing dip bacteriostatic agents.

Generally only one indicator organism is used in a standard testing method. However, as a variety of micro-organisms are of importance in sheep dipping, it is suggested that more than one organism be used. Er. rhusiopathiae and a strain of E. coli would be suitable representatives of Gram-positive and Gram-negative bacteria. Some value would be obtained from the inclusion of one or more fleece micro-organisms also.

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APPENDIX 1

TABLE A1.1

Broth Dilution Turbidimetric Method Results

Organism	Chemical	Concentration of test chemical ($\mu\text{g/ml}$)											Inoc. cont.	Uninoc. cont.	
		5000	2500	1250	625	312	156	78	39	19	9.5	4.8			
<u>E. coli</u>	Arsenic	-	-	-	+	+	+	+	+	+	+	+	+	+	+
	HPMTS	-	-	-	+	+	+	-	-	+	+	+	+	+	+
	ATMTAC	-	-	-	-	-	-	-	-	+	+	+	+	+	+
	LDBAC	-	-	-	-	-	-	-	-	+	+	+	+	+	+
	Water	-	-	-	-	-	-	-	-	+	+	+	+	+	+
			+	+	+	+	+	+	+	+	+	+	+	+	+
<u>E. coli</u> (H_2S)	Arsenic	-	-	-	-	-	-	-	-	-	-	+	+	+	+
	HPMTS	-	-	-	-	-	-	-	-	-	-	+	+	+	+
	ATMTAC	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	LDBAC	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	Water	-	-	-	-	-	+	+	+	+	+	+	+	+	+
			+	+	+	+	+	+	+	+	+	+	+	+	+

- = No growth, + = slight growth, and + = heavy growth.

TABLE A1.1 Continued

170.

Organism	Chemical	Concentration of test chemical ($\mu\text{g/ml}$)											Inoc. cont.	Uninoc. cont.	
		5000	2500	1250	625	312	156	78	39	19	9.5	4.8			
<u>Ps. aeruginosa</u>	Arsenic	-	-	-	-	+	+	+	+	+	+	+	+	+	-
	HPMTS	-	-	-	+	+	+	+	+	+	+	+	+	+	-
	ATMTAC	-	-	+	+	+	+	+	+	+	+	+	+	+	-
	LDBAC	-	-	+	+	+	+	+	+	+	+	+	+	+	-
	Water	+	+	+	+	+	+	+	+	+	+	+	+	+	-
			+	+	+	+	+	+	+	+	+	+	+	+	-
<u>Er. rhusiopathiae</u>	Arsenic	-	-	-	-	-	-	-	-	+	+	+	+	+	
	HPMTS	-	-	-	-	-	-	-	+	+	+	+	+	+	
	ATMTAC	-	-	-	-	-	-	-	+	+	+	+	+	+	
	LDBAC	-	-	-	-	-	-	-	-	-	+	+	+	+	
	Water	+	+	+	+	+	+	+	+	+	+	+	+	+	
			+	+	+	+	+	+	+	+	+	+	+	+	

- = No growth, + = slight growth, and + = heavy growth.

TABLE A1.2 Broth Dilution and Agar Subculture Results

Organism	Chemical	Concentration of test chemical ($\mu\text{g/ml}$)											Inoc. cont.	Uninoc. cont.	
		5000	2500	1250	625	312	156	78	39	19	9.5	4.8			
<u>E. coli</u>	Arsenic	-	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
	HPMTS	-	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
	ATMTAC	-	-	-	-	-	+	+	+++	+++	+++	+++	+++	+++	-
	LDBAC	-	-	-	-	-	+	+	+++	+++	+++	+++	+++	+++	-
	CCPP	-	-	-	-	+	+++	+++	+++	+++	+++	+++	+++	+++	-
	HCP	-	-	-	-	-	-	++	+++	+++	+++	+++	+++	+++	-
	HCP	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
	TCC	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
	TCC	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
	Water	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
	Emulsifier	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
<u>E. coli</u> (H_2S)	Arsenic	-	-	-	-	-	-	++	+++	+++	+++	+++	+++	-	
HPMTS	-	-	-	-	-	-	+	+	+++	+++	+++	+++	+++	-	
ATMTAC	-	-	-	-	+	++	+++	+++	+++	+++	+++	+++	+++	-	
LDBAC	-	-	-	-	+	+++	+++	+++	+++	+++	+++	+++	+++	-	
CCPP	-	-	-	-	+	+	+++	+++	+++	+++	+++	+++	+++	-	
HCP	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	
HCP	+	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	
Water	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	
Emulsifier	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	
Emulsifier	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	

- = No growth, + = slight growth, ++ = medium growth, and +++ = heavy growth.

APPENDIX 2

TABLE A2.1

Results of agar diffusion method. Zone diameters around paper pads containing 120 µg of test chemicals.

Organism	Chemical	Zone Diameters (mm.)					
<u>E. coli</u>	Arsenic	10.6	10.5	10.2	10.1	10.8	10.9
	HPMTS	13.9	13.8	13.8	13.9	14.3	13.5
	ATMTAC	11.0	11.3	11.1	11.4	10.7	10.6
	LDBAC	12.1	11.9	12.7	12.5	12.5	12.8
	CCPP	16.6	16.7	17.5	16.9	16.4	16.8
	HCP	8.7	8.5	8.6	8.8	8.6	8.5
	TCC	6.2	6.2	6.2	6.2	6.2	6.2
	Water	6.2	6.2	6.2	6.2	6.2	6.2
	65% Acet.	6.2	6.2	6.2	6.2	6.2	6.2
<u>E. coli</u> (H ₂ S)	Arsenic	Greater than 40					
	HPMTS	13.2	13.3	12.9	12.8	13.5	13.7
	ATMTAC	9.8	9.7	9.9	9.6	9.6	9.8
	LDBAC	8.4	8.8	8.6	8.9	8.9	9.0
	CCPP	7.4	7.3	7.2	7.3	7.3	7.4
	HCP	7.3	7.2	7.1	7.1	7.3	7.2
	TCC	6.2	6.2	6.2	6.2	6.2	6.2
	Water	6.2	6.2	6.2	6.2	6.2	6.2
	65% Acet.	6.2	6.2	6.2	6.2	6.2	6.2
<u>Ps. aeruginosa</u>	Arsenic	15.6	16.6	16.4	15.2	15.5	15.6
	HPMTS	10.3	10.3	10.5	10.7	10.8	10.5
	ATMTAC	8.8	8.6	8.4	8.5	8.7	8.6
	LDBAC	8.0	7.9	8.1	8.2	8.2	7.9
	CCPP	6.2	6.2	6.2	6.2	6.2	6.2
	HCP	7.8	7.9	7.6	7.5	7.8	7.7
	TCC	6.2	6.2	6.2	6.2	6.2	6.2
	Water	6.2	6.2	6.2	6.2	6.2	6.2
	65% Acet.	6.2	6.2	6.2	6.2	6.2	6.2
<u>Er. rhusiopathiae</u>	Arsenic	19.0	18.4	18.5	19.1	18.9	18.6
	HPMTS	17.5	17.3	17.0	17.2	17.4	17.7
	ATMTAC	22.1	21.2	21.0	20.5	20.8	21.1
	LDBAC	23.2	23.7	22.9	22.6	23.1	23.3
	CCPP	6.2	6.2	6.2	6.2	6.2	6.2
	HCP	20.7	20.1	20.4	20.3	19.6	20.4
	TCC	6.2	6.2	6.2	6.2	6.2	6.2
	Water	6.2	6.2	6.2	6.2	6.2	6.2
	65% Acet.	6.2	6.2	6.2	6.2	6.2	6.2
<u>D. congolensis</u>	Arsenic	17.0	16.9	16.5	16.3	16.6	16.8
	HPMTS	22.9	22.6	22.7	23.7	23.9	24.0
	ATMTAC	23.1	23.0	23.5	23.1	23.2	22.9
	LDBAC	24.3	24.5	25.1	25.0	24.7	24.4
	CCPP	Greater than 40					
	HCP	18.6	18.1	18.5	18.0	18.6	18.4
	TCC	8.8	8.6	8.6	8.9	8.9	9.0
	Water	6.2	6.2	6.2	6.2	6.2	6.2
	65% Acet.	6.2	6.2	6.2	6.2	6.2	6.2

TABLE A2.2

Results of agar diffusion method.
Zone diameters around paper pads
containing 20 μ l of test solution.
D. congolensis. Test chemical CCPP

Amount of chemical per pad	Zone Diameter (mm.)					
20 μ g	15.8	15.7	16.0	16.2	16.3	16.4
10 μ g	11.5	11.2	11.3	11.6	11.4	11.2
65% Acetone	6.2	6.2	6.2	6.2	6.2	6.2

TABLE A2.3

Results of agar diffusion method.
Zone diameters around paper pads
containing 20 μ l of arsenic test
solution. Test organism D. congolensis.

Amount of chemical per pad	Zone Diameter (mm.)					
5 μ g	18.9	18.9	19.6	19.4	19.7	19.4
Water	6.2	6.2	6.2	6.2	6.2	6.2

TABLE A2.4

Analysis of variance table for agar
diffusion test of E. coli

Due to:	Degrees of Freedom	Sums of Squares	Mean Square	F
Blocks	5	0.110		
Treatments	8	680.473	85.059	1296.46**
Error	40	2.624	0.065	
Total	53	683.208		

Standard Error = 0.256

Coefficient of Variation = 2.509

** = Significance at the 1% Level.

TABLE A2.5 Analysis of variance table for agar diffusion test of E. coli (H_2S).

Due to:	Degrees of Freedom	Sums of Squares	Mean Square	F
Blocks	5	0.164		
Treatments	8	9610.760	1201.345	58307.63**
Error	40	0.824	0.020	
Total	53	9611.748		

Standard Error = 0.143

Coefficient of Variation = 1.124

** = Significance at the 1% Level

TABLE A2.6 Analysis of variance table for agar diffusion test of Ps. aeruginosa

Due to:	Degrees of Freedom	Sums of Squares	Mean Square	F
Blocks	5	0.122		
Treatments	8	476.853	59.606	1243.30**
Error	40	1.917	0.047	
Total	53	478.893		

Standard Error = 0.218

Coefficient of Variation = 2.610

** = Significance at the 1% Level

TABLE A2.7 Analysis of variation table for agar diffusion test of Er. rhusiopathiae

Due to:	Degrees of Freedom	Sums of Squares	Mean Square	F
Blocks	5	0.662		
Treatments	8	2701.393	337.674	4650.84**
Error	40	2.904	0.072	
Total	53	2704.960		

Standard Error = 0.269

Coefficient of Variation = 1.933

** = Significance at the 1% Level.

TABLE A2.8 Analysis of variance table for agar diffusion test of D. congolensis

Due to:	Degrees of Freedom	Sums of Squares	Mean Square	F
Blocks	5	0.315		
Treatments	8	8770.160	1096.270	13397.75**
Error	40	3.272	0.081	
Total	53	8773.748		

Standard Error = 0.286

Coefficient of Variation = 1.451

** = Significance at the 1% Level

APPENDIX 3TABLE A3.1

Weights ((g) of clean condition to constant weight at 67% R.H., 22.2° wool samples taken from test animals for assays of Ps. aeruginosa.

Sheep Number	Date			
	29/10/72	30/10/72	2/11/72	4/11/72
26/71	2.4697	1.5741	-	1.6868
34/71	2.2720	1.5506	1.3650	1.5455
38/71	1.9081	1.0497	-	1.8001
20/71	2.5568	1.3399	1.3935	1.5659
25/71	1.8661	1.4738	1.5130	1.4237
53/71	1.4784	1.0597	-	1.3326
49/71	1.9169	0.9030	1.0468	1.8989
58/71	2.1691	1.3405	-	1.4113
79/71	1.5911	0.9011	-	1.3179
4/71	1.5887	1.8059	1.4852	1.5398
29/71	1.6807	1.3099	1.1490	1.6154
78/71	1.1989	1.0921	-	1.5501
6/71	1.3045	1.4712	-	1.6627
74/71	1.2338	1.1373	1.6507	1.2342
82/71	0.9301	1.1662	1.3438	1.5211

- Not weighed because no organisms detected.

TABLE A3.2

Results of presumptive coliform counts.
Numbers of coliforms/100ml dip wash.

Time	Treatment	Most Probable Number	95% Confidence Limits	
			Lower	Upper
1 day after dipping	Water uninoc'd	9.3×10^5	1.5×10^5	3.8×10^6
	Water inoc'd	2.1×10^6	3.5×10^5	4.7×10^6
	Arsenic inoc'd	4.6×10^4	7.1×10^3	2.4×10^5
	LDBAC inoc'd	< 3		
	HCP inoc'd	2.4×10^4	3.6×10^3	1.3×10^5
2 days after dipping	Water uninoc'd	4.3×10^5	7.0×10^4	2.1×10^6
	Water inoc'd	2.4×10^6	3.6×10^5	1.3×10^7
	Arsenic inoc'd	2.4×10^4	3.6×10^3	1.3×10^5
	LDBAC inoc'd	< 3		
	HCP inoc'd	2.1×10^4	3.5×10^3	4.7×10^4
4 days after dipping	Water uninoc'd	1.5×10^6	3.0×10^5	4.4×10^6
	Water inoc'd	4.6×10^6	7.1×10^5	2.4×10^7
	Arsenic inoc'd	4.3×10^3	7.0×10^2	2.1×10^4
	LDBAC inoc'd	< 3		
	HCP inoc'd	2.8×10^3	1.0×10^3	1.5×10^4
7 days after dipping	Water uninoc'd	2.3×10^5	4.0×10^4	1.3×10^6
	Water inoc'd	1.4×10^5	3.0×10^4	3.7×10^5
	Arsenic inoc'd	7.0×10^2	1.0×10^2	2.1×10^3
	LDBAC inoc'd	< 3		
	HCP inoc'd	4.0×10^2	0.5×10^2	2.0×10^3
10 days after dipping	Water uninoc'd	9.0×10^2	1.0×10^2	3.6×10^3
	Water inoc'd	1.1×10^3	3.0×10^2	3.6×10^3
	Arsenic inoc'd	4.3×10^2	7.0×10	2.1×10^3
	LDBAC inoc'd	< 3		
	HCP inoc'd	1.1×10^2	3.0×10	3.6×10^2

Dip washes inoculated with E. coli (H₂S) 2 hours after dipping.