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THE DYNAMICS OF 4,4'-DIAMINODIPHENYL SULFONE ACTION ON POPULATIONS OF SELECTED MYCOBACTERIA, IN VITRO

by

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DISSERTATION

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ABSTRACT

Effects of 4,4'-diaminodiphenylsulfone on Population Dynamics of Selected *Mycobacteria*, *in vitro*.

The studies of inhibition of *M. leprae* in the mouse foot pad by DDS (4,4'-diaminodiphenylsulfone), the drug of choice for leprosy, have raised questions concerning its effects on population dynamics that cannot be answered in that model system.

Because *M. leprae* is not cultivable, alternate model species, *M. kansasii* and *M. smegmatis* 607, were selected on the basis of high susceptibility to DDS, great Adansonian dissimilarity, and the occurrence of *M. kansasii* in human disease.

Populations of bacilli were studied in the absence and presence of DDS and/or PABA (para-aminobenzoic acid), by standard colony counting, tube dilution, and optical density measurement techniques, as well as colony counting modified by use of single-cell inocula and special direct counting techniques. Colonies that appeared in the presence of DDS were analysed for DDS susceptibility and taxonomic characteristics. The dynamics were studied by repeated observation of population size over periods extending up to one month for *M. smegmatis* 607, and to two months for *M. kansasii*. Bacilli exposed to DDS in broth were subcultured onto drug-free agar for colony counts. Data were analysed for difference in population size at the $P = .05$ and $P = .001$ levels. Per cent inhibition,

50% inhibitory concentration (IC50), total inhibitory concentration, growth rate, and rate of appearance of colonies were also analysed.

DDS and MADDS (mono-acetyl-DDS) were assayed fluorometrically in Dubos broth and in bacilli cultured in broth containing DDS. The concentration of DDS in broth is not significantly reduced during six months at 4°C, two months at 37°C, a 15 minute autoclave cycle, nor during growth of _M. kansasii_ cultures. MADDS was not detected in any circumstance. Cultures of _M. kansasii_ take up DDS from broth against a concentration gradient. The amount of DDS bound is dependent on concentration of drug and duration of exposure.

The primary effect of DDS inhibition of the selected _Mycobacteria_ is a concentration-dependent slowing of the rate of growth. This is observed directly in liquid culture, as an increase in per cent inhibition with time, as an increase in the IC50 with time, and as a concentration-dependent delay in the appearance of colonies. This is particularly striking when single-cell inocula are used, as all colonies appear simultaneously in the presence of each concentration of drug. The IC50 is lower by more than ten-fold when DDS inhibition is measured in liquid culture than when it is measured on agar. DDS is bactericidal, but only with very small inocula, and a concentration of drug greater than 100-fold the IC50. Mutants exhibiting a low degree of resistance were encountered, but rarely.

PABA inhibited both _M. kansasii_ and _M. smegmatis_ 607 at 10 and 100 µg/ml respectively. PABA inhibition, like DDS inhibition, results in a slowing of the rate of growth. At sub-inhibitory

concentrations, PABA completely antagonizes the inhibition of DDS when the two drugs are present in approximately equal concentrations. Partial reversal of DDS inhibition is produced by concentrations of PABA as small as 1/80th that of the DDS.

Exposure of bacilli to DDS in broth followed by inoculation onto drug-free agar resulted in a delay in the appearance of colonies. The length of the delay, like the amount of DDS bound, is dependent on the duration of exposure to drug and the concentration of drug to which the bacilli were exposed. This post-exposure inhibition makes DDS a candidate for intermittent chemotherapy.

I dedicate this work to

RENE DUBOS

... after all the medium is the message...

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Lucy J. Higgins, supervisor, instructor and friend, and to Dr. Louis Levy, boss, teacher, editor, and advisor, both personal and academic, for giving me instruction, example, encouragement, advice, and aid in many forms on innumerable occasions beginning almost a decade ago. Their influence on my life is permanent. Lydia Murray and other members of the Leprosy Research Unit, Public Health Service Hospital, San Francisco, I thank for their patience and assistance in providing me with space, equipment, instruction, and materials in facilities already used to capacity. Herman Ng and Kenneth Schilling I thank for invaluable assistance with cultures. Doctors R. M. Featherstone, L. G. Wayne, S. G. Hegeman, and H. D. Landahl I thank for interest and advice during the experimental work. I thank also Sharon Modrick for typing the manuscript under uncomfortable time restrictions. And last but not least, I thank my husband Mark for suffering with me through the writing and rewriting of the manuscript, and for editing material in what is to him almost a foreign language.

CONTENTS

III Methods

CHAPTER I

INTRODUCTION

Members of the genus *Mycobacterium* are responsible for many human diseases. *M. tuberculosis* and *M. leprae* are the most commonly encountered human pathogens in this genus. Pulmonary disease is caused not only by *M. tuberculosis* (68), but also by *M. kansasii* and *M. intracellulare* (97) and rarely by *M. avium*, *M. scrofulaceum*, *M. fortuitum* (68), and *M. abscessus* (88). Diseases of the superficial tissues are caused not only by *M. leprae*, but also by *M. tuberculosis*, *M. marinum* and *M. ulcerans* (97). Mycobacterial disease in children is caused by a wide variety of species, and takes many forms (59).

There are several good drugs available for tuberculosis (68). Dapsone (DDS*; 4,4'-diaminodiphenylsulfone) is the drug of choice for leprosy (96,97). Those drugs that are most useful in the treatment of tuberculosis are not effective for the treatment of some of the diseases caused by other members of the genus. DDS is inhibitory for *M. kansasii* and *M. ulcerans* at low levels (51,74), and *M. ulcerans* infections have been treated with DDS (97). Because the duration of treatment for most of these diseases is prolonged, extending many years for leprosy and at least 2 years for pulmonary disease, it is necessary to explore ways to maximize the antibacterial effectiveness while minimizing the toxicities that are produced by each of the available drugs.

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*Abbreviations are identified in the first table of the appendix.

One means of maximizing effect while minimizing toxicity may be the intermittent rather than continuous administration of the antimicrobial drug (18,34,86). In an attempt to learn the optimal frequency of INH administration in the treatment of pulmonary tuberculosis, clinical trials were designed to compare the standard once- and twice-daily regimens with once and twice weekly dosage schedules (34). According to both bacteriologic and toxicologic criteria, the intermittent regimens were shown to be the more effective. An additional benefit was reported. Patient cooperation improved markedly as the interval between dosages, and therefore clinic visits, was increased. The cost per patient was less, and the health care facilities could adequately care for a larger number of patients.

Studies of experimental tuberculosis in guinea pigs confirmed the bacteriologic findings of the clinical trials (18). The four sets of experimental animals were given the same total amount of drug, but the interval between doses was doubled as the individual dose was doubled. The results indicated that the peak drug concentration achieved in the animal was much more important to the antibacterial action than was the average or the minimum concentration.

Studies *in vitro* of the population dynamics of *M. tuberculosis* and its inhibition by drugs have suggested an explanation of the results of the clinical trials and the experiments in guinea pigs. These studies have also suggested a criterion for the separation of drugs into two categories according to their potential for use in intermittent chemotherapeutic regimens (16,18,19).

Although DDS is known to be inhibitory to several cultivable members of the genus Mycobacterium (51,74), no studies of its effects on population dynamics in vitro have been reported. Studies of these effects have been attempted in the mouse foot pad infection by M. leprae (92,95,98). The interpretation of the results of those studies has been complicated by the relation of the host to the drug-parasite interaction.

It was decided to undertake a study of the effects of DDS on susceptible cultivable Mycobacteria in vitro. Experiments would be addressed to two overlapping problems involving questions of population dynamics. First, does DDS exert effects on mycobacterial populations predictive of its suitability for intermittent chemotherapeutic regimens? Second, does DDS exert effects on mycobacterial populations consistent with the assumptions used in the interpretation of the results in the mouse foot pad experiments?

Questions that will help to answer the above include the following: Is the onset of inhibition immediate or delayed? Is the antimicrobial effect bacteriostatic, bactericidal, or both? Does the degree of inhibition remain constant or does it diminish? To what degree are these effects dependent upon drug concentration? What effects are produced by the metabolic antagonist PABA?

The historical information presented in the next section has been selected to provide both a general framework of knowledge of Mycobacteria, and a more detailed account of the state of knowledge of drug effects as they relate to the questions under consideration.

CHAPTER II

HISTORY

CLINICAL TRIALS

The history of the acceptance of DDS as effective chemotherapy for leprosy has been one of discerning clinical observation. This work was unaided by the usual drug studies <u>in vitro</u> or in experimental animal infections.

This history has been reviewed thoroughly by Chang, Wolcott and Doull (13), by Doull (22) and by Wade (113). Levy (57) has more recently analysed this history with specific emphasis upon the evidence for the efficacy of DDS in the treatment of the lepromatous form of the disease. This form is characterized by the presence of massive numbers of bacilli. A review of the status of leprosy chemotherapy has been presented by Shepard (96). DDS in leprosy prophylaxis has been described by the Fasals and Levy (28).

The highlights of the early work include the first trials of any sulfone published by Faget and his co-workers in 1943 (27). The results of the clinical trials of large doses of parenteral DDS by Cochrane (14) and of smaller oral doses by Lowe (61) both appeared in 1949. The utilization of DDS spread rapidly from that time. However, the first large controlled clinical trial did not appear until 1954 (22). The ways in which the complex disease process of leprosy bear on the conduct and interpretation of clinical trials of chemotherapeutic agents have been discussed in detail by Waters, Rees, and Sutherland (115). The efficacy of DDS has become

so widely accepted (even by patients) that by the early 1960's it had become impossible to include a placebo regimen in a chemotherapeutic trial in lepromatous leprosy (57).

BACTERIAL INDEX

Before 1960, the only available objective measure of successful treatment with an antimicrobial agent was the microscopic examination of smears of the skin lesions stained to demonstrate AFB. In some cases this measure confirmed the clinical impression, but in others, an apparently conflicting result was found. The bacilli remain in the lesions for years after the beginning of DDS therapy, and the number of organisms decreases only gradually (about one log unit per year) (96). Thus, the repeated measurement of the BI did not provide a rapid or sensitive measure of the response to chemotherapy.

MORPHOLOGIC INDEX

Muir (67) had noted that after a few weeks of DDS treatment, the bacilli showed "granulation": and that this progressed to disintegration followed by "absorption", by which he presumably meant disappearance. Lowe (60) had also noted the change in appearance of bacilli from patients who were treated with DDS for an extended period of time. These he described as being "so abnormal in morphology that they appear to be the remains of bacilli disintegrating rather than living bacilli."

These early observations provided the foundation for the MI (84), also termed the "solid ratio", which is the per cent of organisms

observed in preparations stained with the acid-fast stain that are uniformly and brightly stained. Those organisms that appear "solid" are presumed to be viable; all others are presumed non-viable. The decrease of this index during treatment reflects directly the antimicrobial effect of the drug, and is a more sensitive measure of drug effect than is the fall of the BI which only decreases as dead organisms are destroyed or removed. Using the MI, viability of M. leprae was found to decrease from an average pretreatment value of 50% to 10% during the first three months of DDS therapy (114). The validity of this index as a measure of viability has been established by the use of the mouse foot pad method (98,103).

THE MOUSE FOOT PAD METHOD

The laboratory investigation of the effects of drugs on M. leprae became possible in 1960 when Shepard (91) introduced the method of cultivation of the leprosy bacillus in the foot pads of mice. This method was soon employed in Britain (80,81) and Belgium (73).

In this method, a few thousand M. leprae bacilli are injected into the hind foot pads of mice. The mice are maintained at 20°C. for six months at which time the population of M. leprae in each foot pad can be shown by microscopic examination to have reached about 1 million. As there is little multiplication after this, six months marks the beginning of the plateau phase. The inoculum is too small to be detected. Detection of any organisms therefore represents multiplication of the bacilli. When inhibitory

drugs are administered to the animals (mixed in the chow), the bacterial population either fails to reach the level of detectibility during 18 months or reaches the plateau phase more slowly than the control population.

The mouse foot pad method has been effectively used to measure the time course of the reduction of the proportion of viable _M. leprae_ in patients undergoing treatment of lepromatous leprosy (101). Patients with drug resistant bacterial strains have been identified and distinguished from those with treatment failures from other causes (75,78,79,83,93).

The mouse foot pad technique has also been employed to screen anti-tuberculosis drugs and new drugs for activity against _M. leprae_ (99). The minimal effective dosages of the inhibitory drugs have been measured in the mouse. In the case of DDS this was found to be very small, giving DDS treatment of leprosy one of the largest therapeutic indices (104).

By manipulation of the schedule of dosing the experimental mice with drugs, the method has provided information about the effects of the drugs on the population dynamics of _M. leprae_. It has been used to classify drugs as inherently bactericidal or bacteriostatic (92). The promptness of the resumption of multiplication by the surviving bacterial population following the termination of drug exposure has been studied, and delay in the onset of inhibition has been observed.

MYCOBACTERIAL TAXONOMY AND CHARACTERISTICS

The identifying characteristic of the genus *Mycobacterium* is acid-fast staining. Hansen identified *M. leprae* with leprosy a few years before Koch identified *M. tuberculosis* with the diseases it caused. Unfortunately, the identification of "Koch's bacillus" with specific characteristics of growth, pigment, and pathogenicity (123) led to the practice by which *Mycobacteria* were classified as non-pathogenic or as *M. tuberculosis*. That mycobacterial cultures differed from each other was recognized. Association of the differences in cultural characteristics led to designations by variety, such as *M. tuberculosis* var. *hominis*, *M. tuberculosis* var. *bovis*, *M. tuberculosis* var. *avium*, etc. Other strains were considered merely aberrant, and designations such as "anonymous" and "atypical" came into common use. Still other members of the genus continue to be referred to by trivial names such as timothy bacillus, butter bacillus, Battey bacillus and frog bacillus.

An unfortunate result of this taxonomic confusion was that when antibacterial drugs became available, they were frequently tested for their anti-tuberculosis potential on non-pathogenic species (32) merely because the latter were acid-fast. Testing potential anti-leprosy compounds against *M. lepraemurium* (9-11), *M. marinum* (35), or *M. tuberculosis* (21) in experimental disease in mice represents a similar practice.

"Fortunately recent taxonomic studies have brought order into the chaos." (97). Most of the varietal designations have been demonstrated to deserve species standing. Many of the species now

recognized have simply been redesignated by a Latin binomial, although many of them have been reduced to synonomy in the process. The long awaited eighth edition of Bergey's Manual will include approximately 30 species (118).

Much taxonomic work was stimulated by the concept of the "virulence test." Ideally this test distinguishes pathogenic mycobacterial strains from all other acid-fast strains encountered in the clinical laboratory. Although this ideal has not been fulfilled in a single test, a combination of criteria can decide the significance of any strain. Many of the procedures devised as virulence tests have contributed to the battery of useful clinical taxonomic tests and to the Adansonian classification of the entire genus (117).

Mycobacteria have several characteristics which may be of some significance in the design of both therapeutic regimens and experimental procedures. As a group they grow quite slowly compared to most pathogenic bacteria. Most of the species implicated in human disease grow more slowly than non-pathogenic or saprophytic strains. M. tuberculosis has a doubling time of about a day (2); M. ulcerans doubles about once in three days (30). The usual estimate for M. leprae during logarithmic multiplication in the mouse foot pad is one doubling in 12 to 13 days (91). The most rapid growth rates are exemplified by that of M. smegmatis which may double every 4 or 5 hours (33).

Mycobacteria have in common a very high lipid composition. This may have an effect upon drug permeability. It also tends to make the cells in a culture adhere to each other, making quantitative work more difficult.

Nutritionally, there is considerable variation. *M. smegmatis* is a most competent organism. A wide variety of sugars, amino sugars, alcohols, glycols, sugar alcohols, amino acids, acids of intermediary metabolism, and amines can be utilized by it for sources of carbon or nitrogen (109). On the other hand *M. tuberculosis* utilizes glucose alone (71). The growth requirements of *M. leprae* and *M. lepraemurium* are not now known.

Temperatures that permit growth range from 10°C. to 52°C. for *M. phlei*. *M. tuberculosis* and *M. bovis* have a narrow temperature range with a maximum just above body temperature. *M. leprae* shares a characteristic lower temperature requirement with *M. ulcerans*, *M. thamnopheous* and *M. marinum*; none of these grew well at temperatures above 33°C. Higher temperatures are not merely inhibitory; they are lethal. Only one of the approximately 30 mycobacterial species is not killed by 4 hours exposure to 60°C.

Opportunistic pathogenicity appears to be sufficiently common among *Mycobacteria* that, in the laboratory setting, they all must be handled with great respect.

Susceptibility to some drugs has proven to be of value in taxonomic studies. It is clear that drug susceptibility is a species characteristic, and is not genus-wide. In the cases of those drugs which have not become part of taxonomic testing programs, there have been found wide ranges of susceptibility within homogeneous groups. It is interesting that the anti-tuberculosis drug PAS is used taxonomically. Certain species degrade it as a stable characteristic. The failure to find genus-wide patterns of drug susceptibility

explains why testing potential chemotherapeutic drugs against strains other than that causing the disease for which they were proposed may yield erroneous results.

EFFECTS OF DDS ON *MYCOBACTERIA IN VITRO*

Study of the inhibition of cultivable *Mycobacteria* by DDS began in 1940 when Rist and associates studied the effect on *M. avium* (85). This was followed in 1943 by the work of Fitzgerald and Finestone (32), who tested DDS inhibition of *M. smegmatis* 607. At that time this strain was identified as *M. tuberculosis* var. *hominis* 607, but it has been reclassified by Gordon and Smith (39). Fitzgerald and Finestone reported an MIC of 1.5 μg/ml. Youmans and Doub (126,127) tested DDS against *M. tuberculosis* H37Rv by means of a system designed to increase the probability of observing inhibitory effects of newly synthesized compounds. Because of variation from test to test, DDS was included in each series as a standard. Fifty-eight measurements of the MIC of DDS with the same strain, the same medium and standardised inocula gave almost a ten-fold variation, from 3.1 μg/ml to 25 μg/ml. The average MIC was 8.7 μg/ml.

Donovick and his co-workers (20) studied DDS inhibition of BCG, an attenuated strain of *M. bovis*. However, because documentation of the MIC was not the goal of the experiment, the inhibitory drug concentrations were chosen arbitrarily. Naylor and Hanks (69) reported that 5 μg/ml DDS inhibited *M. ranae* and *M. phlei* in liquid culture.

Karlson (51), in 1964, reported a survey of the susceptibility to DDS of over 400 strains of Mycobacterium, representing at least 12 species, including 201 strains of M. tuberculosis. Four concentrations of DDS incorporated in inspissated egg medium were employed: 100 µg/ml, 50, 12.5 and 3.1 µg/ml. Eleven strains were not inhibited by the highest concentration. Seventy-four were inhibited by the lowest concentration employed, suggesting that some of these strains would be inhibited by even lower concentrations of DDS.

This possibility was tested by Pattyn and van Ermengem (74), who employed a three-fold dilution scale to find the MIC's of 68 strains. Fifteen species were represented with an emphasis upon those species that demonstrated the greatest susceptibility in the work of Karlson. Fourteen of their strains were susceptible to 0.3 µg/ml DDS; only one strain was inhibited by 0.1 µg/ml.

Cross resistance between DDS and sulfonamides has been clearly documented by Morrison (66) in studies using M. smegmatis 607.

Medium composition has been reported to influence the results of DDS inhibition studies. Bushby (8), studying inhibition of tubercle bacilli, found 1000 µg/ml were needed for "total effect" when the medium contained 33% blood, whereas only 50 µg/ml were required for complete inhibition when Peizer and Schecter medium was used. Morrison (64) obtained a conflicting result in a study of M. smegmatis 607. He reported that the addition of purified human serum albumin fraction V to the Dubos liquid medium decreased the amount of DDS necessary for a given level of inhibition by four-fold. He suggested that the albumin might potentiate the up-take of DDS by the bacilli.

EFFECTS OF DDS ON M. LEPRAE IN THE MOUSE FOOT PAD

DDS was given continuously mixed in the mouse meal in concentrations of 0.1, 0.01, 0.001, 0.0001, and 0.00001%. It was effective in preventing detectable multiplication of the bacilli at each of these doses for one strain (99,104) while the MIC was 0.0001% for 9 other strains (94).

In subsequent studies the drug was administered to the mice for only selected time periods. An experiment was performed in which DDS at 200 mg/kg was injected into the mice at intervals of 0.5, 1, and 2 months. The data indicated that there was a bacteriostatic effect for 3 weeks following each injection (94). A similar finding was reported by Rees (82). A concentration of 0.1% DDS in the diet administered to mice one day every 14 days suppressed multiplication of M. leprae. From these two experiments it was calculated that slow excretion of the drug accounted for the 14 to 21 days of delay in growth to the plateau phase in excess of that occurring during the period of drug administration (92).

The kinetic method was designed to distinguish bacteriostatic from bactericidal effects. The bacilli would be expected to resume multiplication immediately following termination of exposure to a bacteriostatic drug, whereas a more prolonged recovery would be demonstrated by bacterial populations that had been exposed to bactericidal drugs.

When DDS was administered by the kinetic method early in the logarithmic phase of multiplication, it delayed the growth of the bacilli, but was not able to kill all the members of the

population (92). The delay was of such a duration that, after subtracting 14 to 21 days for slow excretion, there remained 26 to 33 days. Rates of killing were calculated and expressed in units of generation time. However, a ten-fold increase in DDS concentration did not significantly alter the calculated rate of killing.

A second finding resulting from the application of the kinetic method was that the interval that elapsed before DDS was able to stop growth of M. leprae was dependent on the concentration of the drug. However, Shepard states (96) "after growth is stopped bactericidal action seems to proceed at the same rate as at higher dosages." The longest delay (produced by the MIC of 0.0001% DDS in the diet) was one month or about two-and-one-half generation times (93,95). Shepard also states "after only one month of administration there is pure bacteriostasis without bactericide." (96).

When fluorometric methods for the measurement of serum and urinary levels of DDS became available (26,37) the drug concentrations used in these experiments could be translated from % in mouse diet to µg/ml in the mouse serum. The MIC of DDS for M. leprae in the mouse was estimated to be 0.003 µg/ml (104), 0.025 to 0.01 µg/ml (70), or 0.02 µg/ml (93). Thus M. leprae is four to thirty times more susceptible to DDS than most susceptible cultivable strains yet tested. One strain of M. gastri was susceptible to 0.1 µg/ml (74).

EFFECTS OF PABA ON DDS INHIBITION OF MYCOBACTERIA

Various investigators have studied the effect of PABA on inhibition of Mycobacteria by DDS. Fitzgerald and Finestone (32)

determined that 2.5 µg/ml of PABA half reversed the inhibition of *M. smegmatis* 607 by 1.5 µg/ml DDS, the MIC in their system. Donovick (20) and co-workers measured the reversal of DDS inhibition of BCG by PABA. The concentrations of drugs were chosen arbitrarily rather than with relation to the MIC. DDS concentrations were 50, 100, 200, and 400 µg/ml. Growth was measured as per cent transmittance, but the data were reported as per cent inhibition. Inhibition by 50 µg/ml DDS was 78% in the absence of PABA. The reversal by 100 µg PABA/ml of inhibition by the various concentrations of DDS was 81%, 76%, 53%, and 0 respectively. These authors also reported that folic acid did not produce any reversing activity.

Pattyn and van Ermengem (74) studied the ability of PABA to reverse the effects of DDS inhibition of three species susceptible to DDS at 0.3 µg/ml: *M. ulcerans*, *M. kansasii* and *M. gastri*. DDS was employed at concentrations of 3, 10, and 30 µg/ml; PABA was used in 2-fold serial dilutions up to 1:4096. DDS inhibition of *M. ulcerans* was reversed by 0.002 to 0.02 µg PABA/ml. With *M. kansasii* the results were quite different -- 0.9 to 6.8 µg PABA/ml were needed to produce the same degree of antagonism of DDS activity. Inhibition of *M. gastri* by 3 µg DDS/ml was reversed by 6.8 µg/ml PABA. The highest concentration of BABA tested, 13.7 µg/ml, was insufficient to reverse the inhibition caused by 10 and 30 µg DDS/ ml.

The inhibition index is the concentration ratio of drug to the metabolic substrate with which it is believed to compete that produces 50% inhibition (122). The inhibition indices for all of the above studies are as follows: *M. ulcerans*, 500-2000; *M. smegmatis* 607, 332;

M. kansasii, 1-3; BCG, 0.25-2; and *M. gastri*, 0.1-0.5. It is difficult to accept differential permeability as a reasonable explanation of such a range of ratios.

Shepard has tested the ability of PABA to reverse the inhibition by DDS of *M. leprae* in the mouse foot pad (93). The highest subtoxic dose of PABA partially antagonized the antibacterial effect of the minimal effective dose of DDS. With a smaller amount of PABA (i.e. 1%), the inhibition of DDS was less distinct. With ten-fold higher concentrations of DDS, there was no antagonism. The concen- of the chemicals measured in the blood of the mice demonstrated the effective ratio to be 400.

Woods (122) first demonstrated that PABA counteracts the bacteriostatic activity of sulfanilamide. PABA serves as a structural precursor of folic acid (1,55). No evidence has been produced to support the hypothesis that PABA serves any other function (6). The immediate product of the reaction in which PABA serves as a co-substrate is dihydropteroic acid. Brown has shown that this reaction occurs in *M. avium* (6). The enzyme that catalyses the subsequent reaction, in which dihydropteroic acid and glutamic acid react to form dihydrofolic acid, was also found in extracts of *M. avium* (40). If *para*-aminobenzoyl glutamic acid is supplied, dihydrofolic acid can be formed directly (7,105). The importance of such an alternate pathway in *Mycobacteria* is unknown. Sloan and his co-workers (106) have demonstrated the metabolism of PABA by *M. smegmatis* to *para*-aminobenzyl alcohol and that to *para*-hydroxy aniline. It is also known that 30 strains of *Mycobacteria* isolated from soil

all synthesize PABA as well as several other B-vitamins and excrete them into the medium (52).

CHAPTER III

METHODS

MEDIA

Lowenstein-Jensen (L-J) medium (48) in tubed slants* was used for the maintenance of all the stock cultures.

Dubos broth (24) was prepared from the dehydrated base with the Dubos Medium Albumin supplement as directed. Dubos agar was prepared from the dehydrated base and Dubos Oleic Acid Albumin supplement. The terms "broth" and "agar" refer to the Dubos formulations when they stand alone.

Corn Meal Agar was prepared from the dehydrated base and was the preferred agar medium for experiments using M. smegmatis 607.

Glycerol-urea agar was prepared according to Tsukamura (109). It contained glycerol, urea, $MgSO_4$, phosphate buffer and agar.

All agar plates were prepared in sterile disposable 10 x 100 mm petri dishes inscribed with a 13 mm square grid. Tubed media were prepared in 16 x 125 mm screw cap culture tubes.

All media were incubated a minimum of 18 hours prior to use to check for sterility. Plates that were not used immediately were stored at 4°C. wrapped in polyethylene bags. Tubed media were stored at 4°C. with the caps tightened.

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*Commercial sources of supplies are identified in the second table of the appendix.

CULTURES

Strains that were maintained in the collection or used in the screening are listed in the third table of the appendix with the names of the investigators from whom they were received. The majority of the experiments reported here were done using strain 915 of M. kansasii from the laboratory of Dr. Pattyn. The strain of M. smegmatis 607 used in these studies was from Dr. Morrison. These two strains were chosen for the major experimental work because of the documentation of their DDS susceptibility in the literature, and because of the great dissimilarity of these species in Adansonian taxonomy. The production of human disease by M. kansasii was also a consideration.

DRUGS

Dapsone and PABA were dissolved in 95% ethanol for self-sterilization. The stock solutions contained 2.0 mg/ml DDS and 1.0 mg/ml PABA. These solutions were stored at 4°C. Each was diluted as needed using sterile glassware and sterile distilled water or broth. The drug solutions were added to hot agar media after sterilization. For concentrations of drugs greater than 10 µg/ml, the crystalline drug was weighed out and added to the media before sterilization.

PREPARATION OF INOCULA

Inocula of M. kansasii for broth cultures were prepared in one of two ways. Bacterial mass was scraped off well grown L-J slants

with a sterile spatula and placed in a sterile tared 15 x 125 mm screw cap culture tube and weighed. Dubos broth was added aseptically to provide a suspension of 1 mg bacterial mass/ml of broth. This was then suspended by vigorous agitation using a variable speed mixer. Visible clumps were allowed to settle out for about 5 minutes. The suspension was aseptically transferred to a sterile cuvette and the O.D. was measured and recorded.* The second method was to inoculate broth and incubate until growth produced the desired O.D. Because <u>M. smegmatis</u> 607 did not suspend adequately by the first method, all inocula of this strain were prepared by the second method.

Aliquots of these suspensions were used to seed the experimental media. Inocula for plate cultures were prepared as described and diluted to yield approximately 1000 CFU/ml or a 1:10,000 dilution of a suspension of O.D. = 0.100. For measurement of viable units, serial ten-fold dilutions were prepared.

DIRECT COUNTING OF BACTERIA

Slides were prepared for the enumeration of AFB according to the method of Shepard and McRae (105). Commercially available slides bearing three ceramic 1 cm-diameter circles are used. Ten µl of bacterial suspension and ten ul of a fixative-adhesive solution are mixed in each circle and spread evenly over the area on a level board in such a manner that the bacteria are evenly distributed over the entire area. The air-dried slides are subjected to fume fixation

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*Method of measurement is given on page 27.

and are stained with acid-fast stains. The slides are examined with an apochromatic objective using critical illumination and 1250x magnification. To estimate the number of bacteria in a suspension, ten evenly spaced, nonoverlapping fields are counted along the diameter of each of the three circles. To estimate the distribution of cells in clumps, the number of fields is increased such that at least 100 cells were counted in each circle. Calculation of the number of bacilli/ml is done using the number of bacilli counted and the number of fields examined as variables in an equation in which the area of a field and the area of a circle appear as constants.

PREPARATION OF SINGLE CELL INOCULA

Examination of cell suspensions by the above method showed that only a minority of cells occurred singly, whereas the majority occurred in clumps. The distribution of cells in clumps of a typical suspension of M. kansasii of O.D. = 0.100 is shown in Table I. The numbers of colonies that would be expected to form from such a population were calculated for four arbitrarily chosen levels of viability, and are shown in Table 2. It is clear from examination of Table 2 that the degree of clumping exhibited by M. kansasii introduces significant error into the estimation of viability. Therefore the following method was established.

Bacterial mass from an L-J slant was transferred aseptically to a tube containing Hanks' BSS (42) without BSA. The mass was suspended to form a frankly turbid suspension by agitation with a variable speed mixer. A 2.5 ml aliquot of the suspension was

Table 1

CLUMP SIZE BY NUMBER OF CELLS

The composition of clumps is given for a typical suspension of *M. kansasii*. The size category is the number of cells per clump and is shown in the first column. The per centage of clumps in each size category is shown in the second column. The per centage of cells in each size category is shown in the third column. The numbers in parentheses represent the ± 95% confidence limits.*

Number of cells per clump	Per cent of clumps	Per cent of cells
1	59.3(46-79)	19.3(16-24)
2	16.6(11-24)	10.8(8-14)
3	7.3(4-12)	7.1(5-9)
4	3.3(2-6)	4.3(3-6)
5	2.6(1-5)	4.3(3-6)
6	2.0(0-4)	3.9(2-6)
7	1.7(0-3)	3.8(2-5)
8 or more	7.3(4-12)	46.3(39-54)
Total	100.1	99.8

mean = 3.03

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*Method of computation is given on page 25.

Table 2

INFLUENCE OF CLUMP SIZE ON VIABLE COUNTS

The number of colonies that would be expected to form from a population of 1000 clumps is given for the populations described in Tables 1 and 3. In the first column are shown four arbitrarily selected levels of viability. In the second column is shown the number of colonies that would be expected to form from the population in Table 1. In the third column is shown the number of colonies that would be expected to form from the population in Table 3, which was prepared as a single-cell inoculum. The ± 95% confidence limits are shown in parentheses.*

Proportion of viable cells	Number of Colonies	
	typical suspension	single cell inoculum
.5	645(±50)	520(±45)
.25	395(±39)	268(±32)
.10	263(±32)	126(±22)
.01	29(±11)	10(±6)

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*Method of computation is given on page 25.

aseptically transferred with a pipet to a sterile glass syringe fitted with a Swinney membrane filter holder containing a Gelman membrane filter of 5 µ average pore diameter. The fluid was gently pressed thru the filter and the filtrate was collected into a sterile 15 x 125 mm screw cap culture tube. Two filtrates were always prepared with separate syringe assemblies, because occasionally a cell-free filtrate was obtained.

The concentration of cells in each filtrate was counted on circle slides. To avoid contamination of the inoculum suspension, a 0.2 ml aliquot of each filtrate was transferred to a sterile vial containing 0.05 ml of BSS with 0.5% BSA. This procedure was necessary because the presence of BSA was found to reduce the number of cells that would pass thru the filter, while the presence of 0.1% BSA was necessary for the proper adhesion of cells to the slide during the staining procedure.

When single-cell inocula were to be prepared, M. kansasii was grown in the dark on L-J medium for two reasons. Gale (36) has reported that dark-grown cells of M. kansasii are uniformly staining slender rods, whereas those cells that are pigmented following incubation after exposure to light are much broader. Secondly, Schaefer and Lewis (89) have reported that cells grown on oleate rapidly swell and become banded. In either case the cells become too large to pass thru the filter. All cells found in these filtrates were slender and solidly staining.

M. smegmatis was not prepared as a single cell inocula because the organism was too large to pass thru the filter regardless of the conditions of growth.

The distribution of cells in clumps in a typical filtrate is tabulated in Table 3.

ENUMERATION OF COLONY FORMING UNITS

Agar plates were first placed on a level board. Inocula were then dispensed aseptically in aliquots of 0.1 to 0.4 ml per plate in the center of the agar, after which the drops were spread with sterile bent glass rods, each rod being discarded after use on a maximum of five plates with identical inocula. After the inoculum was well absorbed, each plate was placed in a snug-fitting 4½ x 8 inch polyethylene bag which was then sealed, both to prevent contamination of the environment with the organisms and to reduce the rate of evaporation of water from the medium.

All agar plate cultures were incubated at 37°C. Plates were never stacked more than three deep in the incubator.

Colonies were counted using a Quebec dark field colony counter and a hand tally. Each colony was marked on the bag with a ball point pen; those colonies that appeared later were marked with contrasting colors. Periodically, the correctness of the cumulative counts was checked by removing all the marks with ethanol and repeating the entire count.

Because the number of colonies is a discrete variable, it is treated statistically by means of the Poisson distribution (63). Therefore, the S.D. of the number of colonies is equal to its square root, and the 95% confidence limits for a colony count are equal to the number of colonies ± 1.96 times the square root of the number of colonies.

Table 3

CLUMP SIZE OF SINGLE CELL INOCULA

The count of clump composition is given for a typical single cell inoculum of M. kansasii. The size category is the number of cells per clump and is shown in the first column. The per centage of clumps in each size category is shown in the second column. The per centage of cells in each size category is shown in the third column. The numbers in parentheses represent the ± 95% confidence limits.*

Number of cells per clump	Per cent of clumps	Per cent of cells
1	92.8(74-100)	86.5(69-100)
2	7.2(4-11)	13.5(9-19)
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8 or more	0	0
Total	100.0	100.0

mean = 1.07

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*Method of computation is given on page 25.

MEASUREMENT OF RATE OF APPEARANCE OF COLONIES

The rate of appearance of colonies was studied to learn if there was a significant delay dependent either on drug concentration or on the duration of prior drug contact. The maximal number of colonies for each sample was taken as 100%. For each day of observation, the percent of the maximal number of colonies was plotted together with the ± 95% confidence limits. The rate of appearance of colonies was determined to be significantly different for any pairs of plates for which the confidence bands did not overlap.

OPTICAL DENSITY MEASUREMENTS

When the O.D. of stationary cultures was to be measured, the cultures were prepared in 18 x 150 mm Coleman cuvettes or calibrated Kimax test tubes of the same size. The drug solutions were prepared in twice the desired concentration and were dispensed in 5 ml aliquots, and autoclaved. Dubos broth, prepared at twice the normal concentration, inoculated and well mixed, was dispensed into the sterile drug-containing tubes. A sterile syringe pipet was used to deliver the 5 ml aliquots. The final culture volume was 10 ml. The tubes were mixed and incubated immediately.

At the times selected for making O.D. measurements, the cultures were removed from the incubator. Suspension was achieved by shaking each tube with a variable speed mixer. The tubes were allowed to stand for a brief time to permit the bubbles to rise to the surface. Measurements of O.D. at 580 nm were then performed using a Coleman Jr. or a Coleman Sr. spectrophotometer.

When the O.D. of shaken cultures was to be performed, the cultures were set up in 125 ml Erlenmeter flasks to which had been fused 14 mm test tubes as side arms. The drug was added directly to 50 ml of medium in order to obtain the desired drug concentration. Inoculation was performed by introducing small volumes of bacterial suspensions into the flasks aseptically. The flasks were incubated immediately at 35°C. The cultures were shaken at 200 rpm. on a G 10 Gyrotory Shaker. At the times selected for making O.D. measurements, the cultures were tipped into the side arm tube. The measurements were made using a Klett-Summerson Photoelectric Colorimeter with a green filter. This instrument remained in the warm room with the shaker so that no chilling of the cultures occurred.

In each experiment the instrument was balanced using uninoculated broth.

The linearity and comparability of these three instruments were tested using a series of concentrations of barium sulfate freshly prepared from 1% $BaCl_2$ and 1% H_2SO_4.

Because the O.D. is a continuous variable, the bacterial populations measured by O.D. measurements are assumed to be normally distributed. The 95% confidence limits about the mean of replicates was determined using Student's <u>t</u> variable (47). However, because populations of bacteria are not necessarily normally distributed with respect to drug susceptibility, the non-parametric rank-sum test was used to test the null hypothesis that the medians of two populations are identical (47). The rank-sum test does require the assumption of randomness.

Randomness was tested by total "runs". In this test, runs are series of consecutive values all of which fall either above (= n_1) or below (= n_2) the median of that sample. By comparing the number of runs to tabulated critical values for n_1 and n_2 (which need not be equal)(47), one can determine whether a population was randomly selected.

The results of application of Student's *t* test and the rank-sum test were in agreement in all experiments.

INHIBITION TESTING BY RESAZURIN

The resazurin method used was a variation of the quick method for clinical drug susceptibility testing in tuberculosis which was developed by Kubica and Vestal (53). Resazurin is a redox indicator dye that is blue in the reduced form and is oxidized to a pink compound during bacterial growth.

A stock solution of 0.05% resazurin was prepared in well boiled distilled water. This solution was quite stable at room temperature. Twenty-five ml portions of this solution were autoclaved and appropriate volumes were added aseptically to sterile broth to yield a final concentration of 0.00125%. Drugs were added aseptically to aliquots of the broth and the completed media solutions were dispensed in 1.0 ml aliquots into 15 x 125 mm sterile screw cap culture tubes. Sets of test media were inoculated with one drop of a slightly turbid suspension of the bacterial strain. All tubes were incubated at 37°C. and observed daily for color change. The end point was the observation of a strong pink color with no tinge of blue in the

control tube. Drug-containing tubes that changed color simultaneously with the control tubes for each strain were scored as uninhibited; those that remained blue were scored as inhibited or susceptible.

FLUOROMETRIC ASSAY OF DDS AND MADDS

The method used was essentially that of Peters et al (77). An aqueous sample is saturated with ammonium sulfate and extracted with dichloroethane. After the extract is washed with dilute sodium hydroxide, the drugs are next extracted into 2N HCl and back into dichloroethane in the presence of strong alkali. The fluorescent intensity of the final dichloroethane solution is measured at an excitation wavelength of 295 nm and at two emission wavelengths -- 335 and 410 nm. The percent of each drug measured as the other in the standards, and the quantity of each drug in each sample is then calculated by a computer program.

The precision of the method was assessed from 10 duplicate samples. The difference between duplicates averaged 7.8%.

PER CENT INHIBITION

Per cent inhibition was calculated by dividing the difference between mean growth in the presence and absence of drug by the mean growth in the absence of drug, and multiplying by 100. The 95% confidence intervals about the per cent inhibition were calculated directly by using the value represented by the upper limit of the control and the lower limit of the experimental and vice versa.

MOST PROBABLE NUMBER

Viable units are most accurately estimated by colony counting methods. However, occasions arise in which the number of samples for which viability must be estimated is so large that colony counting becomes impractical. MPN estimates the number of viable units in broth culture by distribution of replicate aliquots of ten-fold dilutions of the culture into tubes that are then incubated at 37°C. Each tube is scored for the development of growth observed as turbidity, or for its absence. Standard tabulations were consulted (63) in which the number of turbid tubes at each dilution yields the MPN of viable units in the sample. The 95% confidence limits were calculated according to Taylor (108).

CHAPTER IV

PRELIMINARY EXPERIMENTS

The experiments reported in this chapter have as their subject matter the methods reported in the preceeding chapter. Particularly, they deal with the validity of the methods in cases in which variations on standard procedures were introduced.

STABILITY OF DDS IN DUBOS BROTH AT 4°C. AND 37°C.

One problem that has chronically confronted the investigator of mycobacterial inhibition is that the organisms grow so slowly that the inhibitory drugs may be degraded before experiments can be completed. The following experiment was addressed to this problem.

Replicate tubes were prepared containing DDS 2 and 10 μg/ml in sterile Dubos broth. Half the tubes of each concentration were stored as 4°C. and half at 37°C. Every two weeks for eight weeks, a pair of tubes for each drug concentration was taken from each temperature and assayed. A final set was assayed at six months.

There was no significant reduction in DDS concentration during the first eight weeks. The six-month samples maintained at 4°C. showed reduction of DDS to 87±12% of the original values. The samples maintained at 37°C. for six months showed a reduction in measurable DDS to 60±11% of the original concentrations. It appears that in experiments lasting as long as 96 days there would not be sufficient degradation of DDS to influence the results. Experiments were normally terminated by 60 days. Secondly, DDS was sufficiently

stable at 4°C. that experimental media could be prepared several days to a few weeks in advance of its use.

STABILITY OF DDS IN THE AUTOCLAVE

In some experiments DDS was self-sterilized with ethanol while in others it was added to the medium prior to sterilization. Therefore it became necessary to study the stability of DDS in broth during a sterilization cycle.

Duplicate tubes of 2 µg/ml DDS in Dubos broth were autoclaved for 15 minutes. The quantity of DDS was assayed and was found to be neither significantly nor measurably reduced.

PLATING EFFICIENCY OF SINGLE CELL INOCULA ON GU AGAR

The selection of single cells by filtration allows the speculation that the selected cells may exhibit an altered viability. The GU agar was chosen for the plating efficiency study because it is a minimal medium.

A filtrate of a suspension of <u>M. kansasii</u> was prepared and counted by the method described. Each of four serial ten-fold dilutions was plated on GU agar in inoculum volumes of 0.1 and 0.3 ml with two exceptions; only 0.1 ml was used from the 1:10 dilution and a volume of 0.2 ml was added for the 1:10,000 dilution. Counts of colonies were made on the incubated plates twice weekly until the counts had become constant.

The results are shown in Table 4. The plates inoculated from the 1:100 and 1:1000 dilutions supported suitable numbers of

Table 4

PLATING EFFICIENCY OF SINGLE CELL INOCULA

The number of colonies is given for each plate. The dilution is given in the first column. The volume of inoculum used is given in the second column. The number of colonies expected, calculated from direct counts, is given in the third column. The number of colonies observed is given in the fourth column. The 95% confidence limits are shown in parentheses.

Inoculum		Number of colonies	
Dilution	Volume	Expected	Observed
1:10	0.1(ml)	5349	Uncountable
1:100	0.3	1605	1497(±76)
	0.1	535	539(±45)
1:1000	0.3	160	138(±23)
	0.1	53	53(±14)
	0.1	53	43(±13)
1:10000	0.3	16	8(±6)
	0.2	10	19(±9)
	0.1	5	7(±5)

Plating efficiency is 91.9±15.6%.

colonies for calculations. The number of colonies expected is calculated from direct counts of the number of AFB in the inoculum prior to dilution. The plating efficiency was 91.9 ± 15.6%. The observed number of colonies does not differ significantly from the expected number of colonies.

RATE OF SETTLING OF M. KANSASII IN BROTH

In turbidimetric experiments using rapidly settling organisms, the interval between suspension of the culture and the making of the O.D. measurement must be precisely timed. For cultures in which the specific gravity of the organism is close to that of water, however, the variation that can be introduced by variation in the interval is reduced to negligible levels.

Nine tubes containing 10 ml Dubos broth were prepared. Another set of nine tubes was prepared containing 100 µg/ml DDS in the broth. Both were inoculated with 0.1 ml of a turbid suspension so that the final suspensions were barely turbid. The tubes were incubated, shaken, and analysed by O.D. measurements.

When the O.D. reached 100, the tubes were shaken and incubated an additional 12 hours. At that time, the O.D. measurements were taken with the least possible agitation of the cultures. They were then immediately shaken with the variable speed mixer, and the O.D. was measured again. The differences between the O.D. measurements made before and after shaking were interpreted to be proportional to the amount of settling that had taken place in the previous 12 hours. In Table 5 the individual measurements are given. The settling in

Table 5

O.D. OF CULTURES BEFORE AND AFTER SHAKING

The O.D. is given for a series of replicate broth cultures following 12 hours of incubation without agitation, and again immediately after resuspension. The first pair of columns show the O.D. for drug-free cultures, while the second pair of columns show the O.D. for DDS-containing broth.

Drug-free broth		DDS-containing broth	
Unshaken	Shaken	Unshaken	Shaken
.150	.182	.128	.152
.133	.165	.133	.152
.143	.160	.140	.162
.138	.161	.130	.148
.147	.155	.131	.150
.138	.162	.129	.145
.148	.159	.130	.148
.152	.178	.130	.152
.150	.152	.148	.148

The mean of each of the above series follows with the 95% confidence limits shown in parentheses.

.144	.163	.133	.150
(±.005)	(±.008)	(±.005)	(±.004)

The differences between the shaken and unshaken O.D. for drug-free and DDS-containing broth are .019 and .017.

the drug-free broth was 11.65% whereas that in the DDS-containing broth was 11.33%, indicating that precise timing would not need to be observed, and that the specific gravity of the cells of M. kansasii is close to 1.

RELATION BETWEEN O.D., CELL MASS AND CELL NUMBERS

It was considered desirable to know the relationship between O.D., cell mass and cell numbers. Therefore, the following study was done.

A mass of actively growing M. kansasii was gently scraped off the surface of an L-J slant, and weighed in a tared tube. For each mg of bacterial mass, 1 ml of Dubos broth was added to the tube. The cells were suspended by vigorous agitation with a variable speed mixer. Circle slides were prepared and bacterial numbers were counted by the standard method. The concentration was $1.48 \pm .75 \times 10^8$ cells per ml.

Additional Dubos broth was used to make various dilutions in cuvettes. The results of O.D. measurements, shown in Table 6, were used to construct a graph from which cell concentration could be derived. Similarly, cell numbers could be estimated from an alternate scale on the same axis as cell concentration.

EFFECT OF PREINCUBATION ON THE RESAZURIN METHOD

Kubica and Vestal (53) recommend that the resazurin be added to cultures for the assessment of INH inhibition following 24 to 48 hours of incubation, because sufficient growth to produce color

Table 6

RELATION BETWEEN O.D. AND CELL MASS

The O.D. is given for a series of broth suspensions of known cell mass concentration. The concentration of cells in units of mg/ml is shown in the first column. The observed O.D. is shown in the second column. The third column shows the O.D. that is calculated with respect to the dilution of cell mass.

Concentration of cells in mg/ml	Observed	O.D. Calculated
1.0	.590	(.590)
0.75	.441	.442
0.5	.291	.295
0.375	.206	.221
0.25	.123	.147
0.125	.042	.074

change may occur prior to the onset of inhibition. To test whether or not this precaution is necessary with DDS, the following experiment was done.

Duplicate sets of tubes containing 0, 1, 10, and 100 µg/ml DDS in 1 ml of Dubos broth were inoculated. A duplicate set was inoculated with a strain from each of these species: M. kansasii, M. marinum, M. scrofulaceum, M. gordonae, M. gastri, M. avium, M. intracellulare, M. terrae, M. fortuitum, M. smegmatis, and M. phlei. Resazurin was added to one set immediately. Both sets were incubated until the resazurin control culture changed color. At that time resazurin was added to the second set of test cultures for that strain. Incubation was again continued to the end point of color change in the control tube. The results in the two sets were identical for all 11 strains, suggesting that DDS does not allow sufficient growth prior to the onset of inhibition to give false readings of resistance.

EFFECT OF INOCULUM SIZE IN THE RESAZURIN METHOD

The influence of inoculum size on the resazurin method of testing for DDS susceptibility was examined using the strain of M. kansasii that was inhibited by 1, 10, and 100 µg/ml DDS in the previous experiment. The usual barely turbid suspension was prepared and a series of three ten-fold dilutions of it were prepared in sterile broth. Each of the four inoculum suspensions was inoculated into a resazurin drug series containing the same drug concentrations as in the preceeding experiment. The tubes were incubated and

observed. The results were identical among the four series, although the smaller inocula required proportionately longer incubation periods to reach the end point. Inoculum size did not influence the apparent susceptibility to DDS over a ten-thousand-fold range of inoculum size.

EFFECT OF DILUTION ON CLUMP SIZE

The possibility that dilution would change the CFU composition was considered and tested. M. kansasii was prepared in a 1 mg/ml suspension in Dubos broth, and 1:10 and 1:100 dilutions were prepared. Circle slides were prepared, and bacterial numbers and clump composition were counted. The results are shown in Table 7 for the suspensions of 1 and 0.1 mg/ml. In the 1:100 dilution, the cell concentration is too small for accurate counting. The mean number of cells per clump for the suspension of 1 mg/ml was 4.09 with 95% confidence limits of 3.5 and 4.8. The mean number of cells per clump for the suspension of 0.1 mg/ml was 3.06 with 95% confidence limits of 2.3 and 4.2. There were no significant differences in CFU composition between the two dilutions.

CHANGES IN CLUMP SIZE DURING INCUBATION

It was considered possible that during incubation clumps would increasingly represent pairs of cells that had divided, but had continued to adhere to each other. Under such circumstances, clumps composed of two, four, and eight cells should become more common. In order to explore this possibility, a suspension of M. kansasii

Table 7

EFFECT OF DILUTION ON CLUMP SIZE

The count of clump composition is given for suspensions of 1 mg/ml and 0.1 mg/ml *M. kansasii*. The size category is the number of cells per clump and is shown in the first column. The per centage of clumps in each size category is shown in the second column for the 1 mg/ml suspension and in the fourth column for the 0.1 mg/ml suspension. The per centage of cells in each size category is shown in the third column for the 1 mg/ml suspension and in the fifth column for the 0.1 mg/ml suspension. The numbers in parentheses represent the ± 95% confidence limits.

Number of cells per clump	1 mg/ml Per cent of clumps	1 mg/ml Per cent of cells	0.1 mg/ml Per cent of clumps	0.1 mg/ml Per cent of cells
1	55.0(43-70)	13.5(11-16)	56.4(35-89)	18.4(12-26)
2	20.8(15-28)	10.2(8-12)	19.8(9-36)	12.9(8-19)
3	4.2(2-7)	3.1(2-4)	5.0(1-11)	4.9(2-8)
4	2.8(1-5)	2.7(2-4)	5.9(1-14)	7.8(4-12)
5	2.5(1-5)	3.1(2-4)	2.0(0-6)	3.2(1-6)
6	1.1(0-2)	1.6(1-2)	1.0(0-4)	1.9(0-4)
7	0.8(0-1)	1.4(1-2)	0	0
8 or more	12.8(8-18)	64.4(57-72)	10.0(3-20)	50.8(38-66)
Total	100.0	100.0	100.1	99.9

was prepared by the standard method. A sample was removed from which a circle slide was prepared. The remainder of the culture was incubated at 37°C. Additional circle slides were prepared from samples taken daily for four days. Bacterial numbers and clump composition were counted by the standard method. The results are shown in Tables 8, 9, and 10. Table 8 shows the per cent of clumps occurring in each size category. Table 9 shows the per cent of cells occurring in each size category. Table 10 shows the actual numbers of clumps in each size category for three replicate samples obtained on day three of incubation.

The per cent of CFU falling in each size category does not vary significantly during four days of incubation. The same is true of the per cent of cells falling in each clump size category, with the following exceptions. A significantly greater per cent of cells falls in the single cell clump class after one day of incubation than at any other time interval, and a significantly smaller proportion falls in that category following four days of growth. A significantly greater per cent of quartets is present following two days of growth than following shorter or longer incubations. There appears to be a smaller proportion of cells in clumps of five, six, or seven cells initially compared to later, but the number of clumps of this size is too small to permit valid comparisons. Finally the number of cells present in clumps of eight or more appears to increase from day 1 to day 4. This trend is reflected in the calculated mean number of cells per clump, which was 5.1 for day 0 (with ± 95% confidence limits of 4-7), 2.34(2-3) for day 1, 3.8(3-5) for day 2, 5.8(5-7) for day 3, and 6.8(6-8) for day 4.

Table 8

CHANGES IN CLUMP SIZE DURING INCUBATION

PART I, PER CENT OF CLUMPS IN EACH SIZE CATEGORY

The composition of clumps is given for a culture of M. kansasii during a four-day incubation. The size category is the number of cells per clump and is shown in the first column. The percentage of clumps in each size category is shown in a column for each sampling period. The numbers in parentheses represent the ± 95% confidence limits.

Number of cells per clump	Day of incubation				
	0	1	2	3	4
1	56.5 (37-85)	64.4 (47-87)	44.0 (31-61)	43.4 (27-61)	34.5 (25-46)
2	22.9 (12-38)	18.7 (11-28)	25.5 (16-37)	21.2 (13-32)	21.4 (15-30)
3	6.6 (1-14)	4.8 (2-9)	3.2 (1-6)	8.5 (4-15)	9.0 (5-14)
4	6.6 (1-14)	3.4 (1-7)	12.0 (6-19)	5.6 (2-11)	6.8 (4-11)
5	0.8 (0-3)	2.4 (0-5)	4.6 (2-9)	3.5 (1-7)	7.4 (4-12)
6	0 (0-0)	0.9 (0-3)	0.5 (0-2)	5.0 (2-9)	2.2 (0-4)
7	0.8 (0-3)	0.5 (0-2)	2.3 (0-5)	1.5 (0-4)	2.8 (1-5)
8 or more	5.7 (1-12)	4.8 (2-9)	7.9 (4-13)	11.1 (6-18)	15.8 (10-23)
Total	99.9	99.9	100.0	98.8	99.9

Table 9

CHANGES IN CLUMP SIZE DURING INCUBATION

PART II, PER CENT OF CELLS IN EACH SIZE CATEGORY

The composition of clumps is given for a culture of *M. kansasii* during a four-day incubation. The size category is the number of cells per clump and is shown in the first column. The per centage of cells in each size category is shown in a column for each sampling period. The numbers in parentheses represent the ± 95% confidence limits.

Number of cells per clump	Day of incubation 0	1	2	3	4
1	11.0 (8-15)	27.5 (21-35)	11.5 (9-15)	7.5 (6-10)	5.1 (4-6)
2	8.9 (6-12)	16.0 (11-21)	13.3 (10-17)	7.3 (5-9)	6.3 (5-8)
3	3.8 (2-6)	6.2 (3-9)	2.5 (1-4)	4.4 (3-6)	4.0 (3-5)
4	5.1 (3-7)	5.7 (3-9)	12.6 (9-16)	3.8 (2-5)	4.0 (3-5)
5	0.8 (0-2)	5.1 (3-8)	6.1 (4-8)	3.1 (2-4)	5.5 (4-7)
6	0 (0-0)	2.5 (1-4)	0.7 (0-2)	5.2 (4-7)	1.9 (1-3)
7	1.1 (0-2)	1.4 (0-3)	4.2 (2-6)	1.8 (1-3)	2.9 (2-4)
8 or more	69.2 (58-82)	35.3 (28-45)	49.0 (41-58)	66.8 (59-76)	70.2 (64-77)
Total	99.9	99.9	99.9	99.9	99.9

Table 10

CLUMP COMPOSITION OF *M. KANSASII*; DAY 3

The composition of clumps is given for a culture of *M. kansasii* following three days of incubation. The size category is the number of cells per clump and is shown in the first column. The actual number of clumps occurring in each size category is shown in a column for each of three replicate samples.

Number of cells per clump	Replicate sample number 1	2	3
1	31	23	32
2	11	17	14
3	7	2	8
4	5	3	3
5	1	5	1
6	2	5	3
7	2	0	1
8 or more	8	4	10
Counts of 8 or more	10,10,8 8,23,16 8,10	11,415, 9,13	13,18,9,27 8,20,30,20, 12,68
Total cells	218	578	351
Total clumps	67	59	72

The conditions of the experiment suggest an explanation for these differences. First, the day 0 observations were made on a mass of surface growth from L-J medium, suspended in Dubos broth which contains Tween 80$^{(R)}$. The increase in the per centage of single cells during the first day of incubation could have resulted from a more thorough penetration of the Tween 80 into clumps than was accomplished during the initial agitation. Contributing to this would be the rapid swelling of cells as the oleate in the Tween is accumulated by the bacteria (89). This would also explain the low values on day 1 for large clumps, and for the mean number of cells per clump. Second, the larger clumps occurred after incubation periods which are sufficiently long that active hydrolysis of the Tween 80 by M. kansasii would have reduced its effective concentration (116).

Although the numerical data are not tabulated here, growth did occur during this experiment. There was no net increase in numbers during the first observation interval. During the succeeding three intervals, bacterial multiplication proceeded at the rate of one doubling for every 15.5 hours.

EFFECT OF FREQUENCY OF SHAKING ON THE RATE OF GROWTH

To examine the dependence of the growth of M. kansasii on agitation, the following experiment was done. Four suspensions of M. kansasii were prepared by serial ten-fold dilution and used to inoculate ten replicate tubes containing DDS in the following concentrations: 0, 0.1, 1.0, 10, and 100 µg/ml in 10 ml Dubos broth.

The tubes were incubated at 37°C. and shaken immediately prior to measurement of O.D. at intervals of 4, 12, 18, 24, and 32 hours, which were repeated up to three times in differing orders. The difference in O.D. for each measurement period was divided by the number of hours in that measurement period.

Growth occurred in measurable amounts in all groups except the lowest inoculum size groups containing 1, 10 and 100 µg/ml DDS. The intervals between shaking correlated inversely with the growth rate during the interval whether drug was present or not. The correlation coefficient for each group was -0.98, -0.99 or -1.0. The correlation coefficient for all groups was -0.99. Therefore, there is an exact dependence of growth rate on aeration both in inhibited and in uninhibited cultures.

CHAPTER V

EXPERIMENTAL RESULTS

INHIBITION BY DDS, SURVEY WITH RESAZURIN

A survey of DDS susceptibility with the resazurin method was made to see how much agreement there was between this method and the findings of other workers by other methods. Two series of tests were completed. In the first series, DDS concentrations of 1, 10, and 100 µg/ml were tested. In the second series, additional strains of species which were found to be susceptible in the first series were tested against DDS concentrations of 0.3, 1, 3, and 10 µg/ml.

The results of this survey are presented for series 1 in Table 11, and for series 2 in Table 12. In the first series, three species exhibited susceptibility to 1 µg/ml DDS: M. kansasii, M. gordonae and M. gastri. All the other species were not susceptible to 100 µg/ml with the exception of M. terrae which was susceptible to 100 µg/ml but not to 10 µg/ml. In the second series, three strains each of M. gastri and M. gordonae were tested together with M. smegmatis 607. In this series, M. gordonae W986 and M. smegmatis 607 were not susceptible to 0.3 µg/ml but were susceptible to 1 µg/ml. The other five strains, three of M. gastri and two of M. gordonae were susceptible to 0.3 µg/ml DDS.

INHIBITION OF M. KANSASII COLONY FORMATION BY DDS ON COMPLEX AND MINIMAL MEDIA -- A PRELIMINARY EXPERIMENT

The possibility that Dubos medium contains an anti-inhibitory factor was considered; therefore, the response to DDS was compared

Table 11

RESAZURIN SURVEY OF DDS SUSCEPTIBILITY

The susceptibility of various strains of Mycobacteria are shown for three concentrations of DDS tested by the resazurin standard method. the name of the species is shown in the first column, followed by the number of strains tested in parentheses. The response is shown in a column for each tested DDS concentration. G (for growth) indicates no inhibition. I (for inhibited) indicates the strains were susceptible.

Species (# of strains)	DDS concentration ug/ml 1	10	100
M. kansasii (4)	I	I	I
M. marinum (3)	G	G	G
M. scrofulaceum (5)	G	G	G
M. gordonae (2)	I	I	I
M. gastri (1)	I	I	I
M. avium (3)	G	G	G
M. intracellulare (3)	G	G	G
M. terrae (1)	G	G	I
Group III unidentified (1)	G	G	G
M. fortuitum (3)	G	G	G
M. smegmatis (2)	G	G	G
M. phlei (2)	G	G	G
Group IV unidentified (3)	G	G	G
M. nonchromogenicum (1)	G	G	G

Table 12

RESAZURIN TEST OF SUSCEPTIBLE *MYCOBACTERIA*

The levels of inhibition of various strains of *Mycobacteria* are shown for four concentrations of DDS tested by the resazurin standard method, series 2. The name of the species is shown in the first column, and is followed by the number of strains tested in parentheses. The response is shown in a column for each tested DDS concentration. G (for growth) indicates no inhibition. I (for inhibition) indicates susceptibility.

Species (# of strains)	DDS concentration µg/ml 0.3	1	3	10
M. gordonae (2)	I	I	I	I
M. gordonae W986 (1)	G	I	I	I
M. gastri (3)	I	I	I	I
M. smegmatis 607 (1)	G	I	I	I

using Dubos agar and the minimal GU agar, using a strain of M. kansasii shown to be susceptible to 1 µg/ml DDS in the survey by the resazurin method.

Duplicate GU and Dubos agar plates were prepared containing DDS in the following concentrations: 0, 0.1, 0.3, and 1.0 µg/ml. A single cell inoculum was prepared by the method described. Inoculum volume was 0.1 for every agar plate. Colonies were counted and recorded twice weekly. The results of this preliminary experiment are presented in Tables 13-16. Tables 13 and 15 show the number of colonies formed on Dubos and GU agars respectively, whereas Tables 14 and 16 show the per cent of maximal colony formation.

An exceedingly steep dose-response curve could be constructed from these results, with one drug concentration producing zero inhibition and the next, three-fold higher, producing 100% inhibition. These data suggest that the response of M. kansasii to DDS is a slowing of the growth rate rather than all-or-none bacteriostasis or bactericide, and that examination of the rate of colony appearance may yield useful information. Even the lowest concentration of DDS used here produced some inhibitory effect, and therefore a lower concentration was examined (see next experiment).

Dubos agar allows more rapid appearance of colonies than the GU agar, both in the absence and presence of DDS. There is no evidence from this experiment that there is any difference in the level of inhibition produced by DDS that would be attributable to the composition of the media.

Table 13

NUMBER OF COLONIES FORMED ON DUBOS AGAR

The number of colonies present on each plate is shown for each observation period in which there were new colonies of *M. kansasii*. The day of the experiment on which new colonies were present is shown in the first column. The number of colonies on each plate is shown in a column for each drug concentration. The ± 95% confidence limits are shown in parentheses.

Day	DDS µg/ml			
	0	0.1	0.3	1.0
13	60(15)	0	0	0
	65(16)	0	0	0
17	61(15)	50(14)	0	0
	67(16)	53(14)	0	0
20	61(15)	60(15)	0	0
	67(16)	61(15)	0	0
44	61(15)	60(15)	40(12)	0
	67(16)	61(15)	66(16)	0

The final observation was made on day 50.

Table 14

PER CENT OF COLONIES FORMED ON DUBOS AGAR

The per cent of the maximal number of colonies of M. kansasii on each plate is shown for each observation period in which there were new colonies. The day on which new colonies were present is shown in the first column. The per cent of the maximal number of colonies for each plate is shown in a column for each concentration of DDS. The ± 95% confidence limits are shown in parentheses.

Day	DDS µg/ml			
	0	0.1	0.3	1.0
13	98(59-163)	0	0	0
	97(59-159)	0	0	0
17	100(60-165)	83(48-142)	0	0
	100(61-163)	87(51-146)	0	0
20	100(60-165)	100(60-167)	0	0
	100(61-163)	100(61-165)	0	0
44	100(60-165)	100(60-167)	100(53-185)	0
	100(61-163)	100(61-165)	100(61-164)	0

The final observation was made on day 50.

Table 15

NUMBER OF COLONIES FORMED ON GU AGAR

The number of colonies present on each plate is shown for each observation period in which there were new colonies of M. kansasii. The day of the experiment on which new colonies were present is shown in the first column. The number of colonies on each plate is shown in a column for each drug concentration. The ± 95% confidence limits are shown in parentheses.

Day	DDS μg/ml			
	0	0.1	0.3	1.0
17	50(14)	0	0	0
	45(13)	0	0	0
20	58(15)	0	0	0
	47(13)	0	0	0
29	63(16)	76(17)	0	0
	52(14)	96(19)	0	0

The final observation was made on day 50.

Table 16

PER CENT OF COLONIES FORMED ON GU AGAR

The per cent of the maximal number of colonies of M. kansasii on each plate is shown for each observation period in which there were new colonies. The day on which new colonies were present is shown in the first column. The per cent of the maximal number of colonies for each plate is shown in a column for each concentration of DDS. The ± 95% confidence limits are shown in parentheses.

Day	DDS µg/ml			
	0	0.1	0.3	1.0
17	79(45-136)	0	0	0
	79(48-152)	0	0	0
20	92(54-155)	0	0	0
	90(52-158)	0	0	0
29	100(59-168)	100(63-157)	0	0
	100(58-173)	100(67-149)	0	0

The final observation was made on day 50.

INHIBITION OF COLONY FORMATION BY DDS

The previous experiment was repeated using GU agar and additional concentrations of DDS. A larger inoculum concentration was used as well.

GU agar plates were prepared containing DDS in the following concentrations: 0, 0.1, 0.3 and 0.5 µg/ml.

A single-cell inoculum was prepared and inoculum volumes of 0.1, 0.2, and 0.3 ml were spread on each of the replicate agar plates.

The incubated plates were observed every day or on alternate days. The day that pin-point colonies became visible was recorded. Colonies were counted when they were approximately 1 mm in diameter, and again on the final day of the experiment -- day 60.

The results of this experiment are shown in Tables 17 and 18, and Figure 1. Table 17 shows the number of colonies that formed on each plate. Table 18 contains the results of various calculations based on the data in Table 17. These include the number of colonies per ml of inoculum, the per cent inhibition, the number of days of delay before the appearance of colonies, and the MIC. The per cent inhibition was either zero or 100% in all cases except that of the plates containing 0.3 µg/ml. The number of colonies formed on these plates represented 33% inhibition. Figure 1 is a graph of per cent inhibition versus logarithm of dose. The IC50 is derived by interpolation of this graph. The curves display two noteworthy aspects. First, they are exceedingly steep. Second, the location and shape of a dose-response curve in this system are dependent upon the time of observation.

Table 17

INHIBITION OF COLONY FORMATION

The number of colonies is given for each plate. The volume of inoculum used is shown in the first column. The number of colonies that appeared is shown in a column for each concentration of DDS. The ± 95% confidence limits for the total number of colonies on each plate is shown in parentheses. The day on which the colonies appeared was different for each drug concentration and is shown in a separate line at the bottom.

Inoculum volume ml.	DDS in µg/ml 0	0.1	0.3
0.1	170(26)	189(27)	113(21)
	206(28)	174(26)	121(22)
0.2	318(35)	397(39)	251(31)
	-	-	237(30)
	-	-	246(31)
0.3	529(45)	501(44)	358(37)
	546(46)	578(47)	343(36)
Day appeared	14	20	41

The final day of observation was day 60.

Table 18

SUMMARY OF INHIBITION OF COLONY FORMATION

The number of colonies per ml of inoculum is given for each drug concentration in the first line. The per cent inhibition calculated from these data is shown in the second line. The ± 95% confidence limits are shown in parentheses. The number of days of delay is shown in the third line. The data are shown in a column for each concentration of DDS.

	DDS in µg/ml		
	0	0.1	0.3
# colonies per ml	1769(180)	1839(183)	1192(149)
% Inhibition	0(0-10)	0(0-15)	33(16-46)
Days of delay	0	7	27

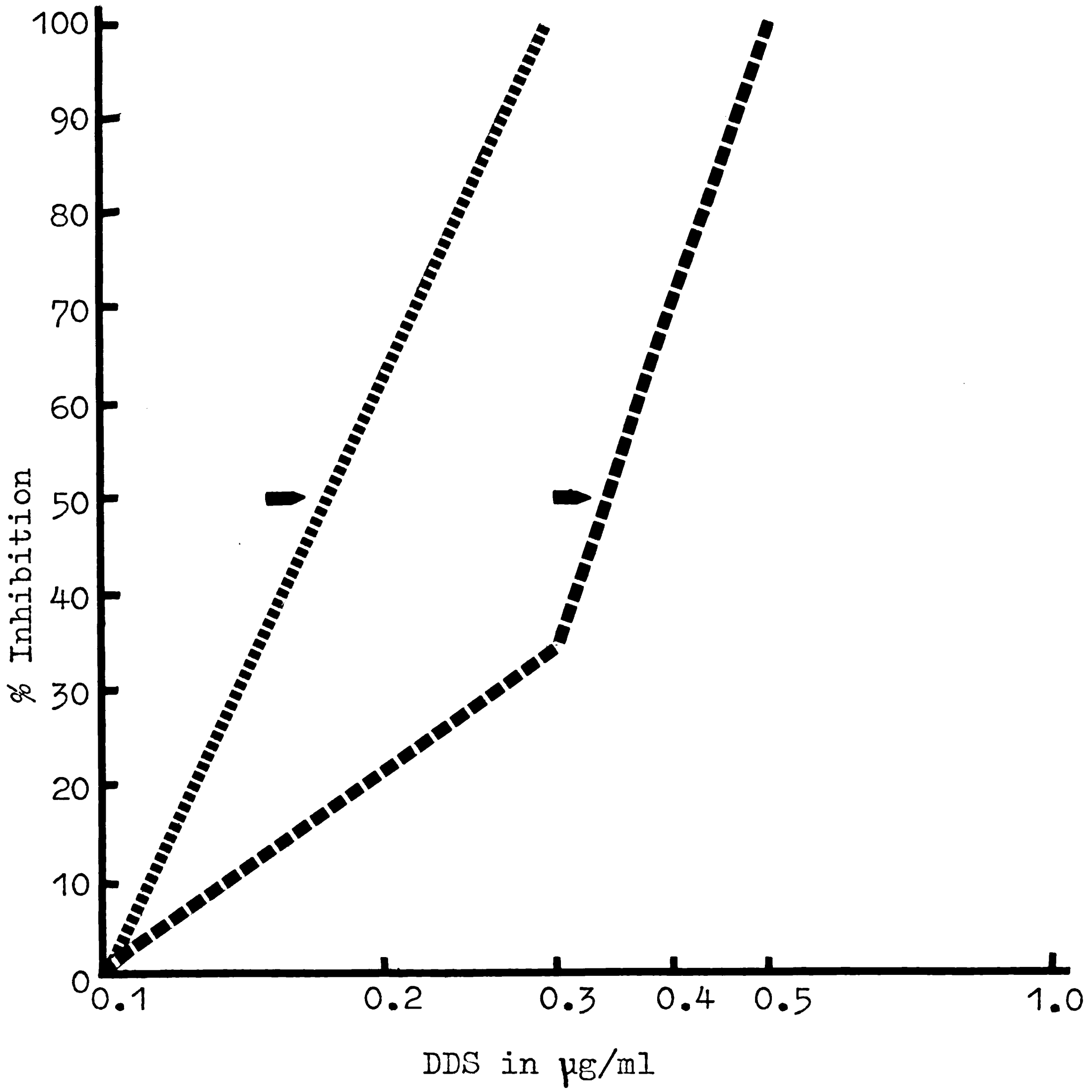
The MIC of DDS for M. kansasii colony formation is shown for each observation period at which new colonies appeared. The IC50 is derived by interpolation on the log dose/% inhibition graph in Figure 1.

Day	IC50		Total inhibition	
	µg/ml	nM	µg/ml	nM
14	--	--	0.1	0.4
21	0.175	0.7	0.3	1.2
41	0.345	1.38	0.5	2.0

Figure 1

INHIBITION OF COLONY FORMATION

Per cent inhibition is plotted on the ordinate. The concentration of DDS is plotted on a logarithmic scale on the abscissa. The interpolated IC50 is indicated. (▮▮▮▮▮▮▮)-- 20-day data. (■■■■)-- 41-day data.



Total inhibition was produced by 0.1 µg/ml DDS for a period of seven days following the appearance of colonies on the plates that did not contain drugs. After colonies appeared on 0.1 µg/ml DDS, there was no inhibition by that concentration. Total inhibition was produced by 0.3 µg/ml for a period of 20 days, following the appearance of colonies on the agar containing 0.1 µg/ml. The plates containing 0.5 µg/ml DDS never developed colonies. The final day of observation was day 60.

The expected number of colonies per ml of inoculum calculated from the count of the filtrate was 1844. The plating efficiency for this experiment was therefore 95.9%.

Because 92% of the CFU in the M. kansasii inoculum were composed of single cells, whereas the remainder were composed of pairs, and because there was no change in count on any plate between the initial and final counts, these data provide a most rigorous measure of the MIC. Measurements of the MIC as total inhibition was discussed in the preceeding paragraph. The MIC as the IC50 was 0.7 nM from day 21 to day 40 and 1.38 nM from day 41 to the termination of the experiment on day 60. No measurement of IC50 was possible prior to the appearance of colonies on the plates containing the lowest concentration of drug, because no point representing zero drug concentration exists on the scale representing the logarithm of dose.

The simultaneous appearance of colonies on the drug containing plates after an interval, the length of which depends upon the drug concentration, following the appearance of colonies upon drug-free agar, can be interpreted in terms of the effects of DDS on bacterial

dynamics. Either DDS causes a slowing of the growth rate, or it causes a prolongation of the lag phase which is then followed by growth at the normal rate. A pattern reflecting a partial contribution of these two effects is also possible. However, there is nothing in this experiment from which one could rule out the possibility that the failure of colonies to appear on the agar plates containing 0.5 µg/ml DDS resulted from a bactericidal effect.

EFFECT OF INCREASED INOCULUM SIZE ON INHIBITION OF COLONY FORMATION BY *M. KANSASII*

The possibility that increased inoculum size could effectively antagonize the inhibition of DDS was studied in this experiment. GU agar plates were prepared containing DDS in the following concentrations: 0, 0.5, 1.0, 1.5, 2.0, 3.0, and 10.0 µg/ml. A single cell inoculum was prepared which contained about 60,000 cells/ml. A 1:10 dilution was prepared with sterile broth. Replicate plates were inoculated with inoculum volumes of 0.1, 0.2, 0.3 and 0.4 ml of each suspension. The incubated plates were observed every day or on alternate days for 60 days.

No colonies of AFB appeared on any of the plates containing DDS during the entire observation period.

THE EFFECT OF DDS ON THE VIABLE NUMBERS AND TURBIDITY IN SHAKEN CULTURES OF *M. KANSASII*

In order to demonstrate whether DDS has a bactericidal effect on *M. kansasii*, the following experiment was done. Selected portions

of the experiment were performed with both MPN tube-dilution and colony count assays of viability. O.D. measurements were made to determine whether total numbers varied with viable numbers.

Erlenmeyer flasks with cuvette side-arms were prepared containing 50 ml. Dubos broth with DDS in the following concentrations: 0, 0.3, 1, 3, and 10 µg/ml. An inoculum of M. kansasii was grown to an O.D. of 0.060. Each flask was inoculated with 1.5 ml of this suspension. The flasks were incubated according to the method described for shaken cultures. The O.D. was measured daily for ten days and on days 15, 17, and 22. Flasks in which turbidity exceeded 250 Klett units were terminated. On days 0, 1, 2, 3, 4, 5, 7, 9, 15, 17, and 22, each flask was sampled to tube-dilution assays of viable numbers. On days 0, 3, 9, 17, and 22, aliquots of the dilutions were plated on Dubos agar, one set containing 1 µg/ml PABA and an identical set containing 1 µg/ml DDS.

The tube-dilution assays were performed in Dubos broth containing 1 µg/ml PABA. Each dilution was performed with a fresh pipet and all were shaken by hand. A 0.1 ml sample was removed from each flask and diluted to 10 ml. If the Klett measurement was equivalent to an O.D. greater than .010, an additional 1:100 dilution was made, and if the O.D. was greater than .100 a further 1:10 dilution was made. From that point all samples were treated identically. The initial 1:100 dilution was followed by four 1:10 dilutions. Each of these five dilutions was distributed in 1.5 ml aliquots, incubated and scored from MPN.

The results of this experiment are presented in Tables 19-21 and

Figure 2. The effect of the various concentrations of DDS on MPN is shown in Table 19; the effect on CFU is shown in Table 20; and the effect on turbidity is shown in Table 21, and Figure 2. It can be seen that MPN and CFU agree quite closely. When the turbidity measurements are converted to concentration of cells (Table 21) the estimate from turbidity always falls within the confidence limits around the measurement of viable numbers. Therefore, essentially all of the cells were viable. No partial bactericidal effect could be demonstrated under these conditions of drug and inoculum concentration. Reduction of growth rate is the effect exerted by DDS, although a small contribution to inhibition from prolongation of the lag phase seems to be taking place.

EFFECT OF INOCULUM SIZE ON INHIBITION BY DDS IN BROTH

Fifty tubes were prepared containing each of the following concentrations of DDS in Dubos broth: 0, 0.1, 1.0, 10, and 100 µg/ml. Four inocula of M. kansasii were prepared by serial ten-fold dilution. Ten tubes from each drug concentration were inoculated with each inoculum. The tubes with the largest inoculum size were turbid, with an O.D. = .040. The cultures with the largest inoculum were observed for increase of turbidity; the others were observed for appearance of turbidity.

Appearance of turbidity in cultures containing DDS at the same time or shortly after the appearance of turbidity in control cultures was interpreted to mean that virtually the entire population had multiplied. From experiments using the method of MPN for viability

Table 19

EFFECT OF DDS ON MPN

The MPN of viable units (x 10^{-6}) is shown for each drug concentration at each observation period. The observation period is shown in the first column. The MPN of viable units is shown in a column for each drug concentration. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml				
	0	0.3	1.0	3.0	10.0
0	1.4 (.36-3.6)	1.4 (.36-3.6)	0.52 (.14-1.4)	3.1 (.81-8.1)	0.87 (.23-2.3)
1	8.7 (2.3-23)	5.3 (1.4-14)	3.3 (.86-8.6)	2.3 (.6-5.9)	0.33 (.08-.86)
2	0.9 (.23-2.3)	3.6 (.94-9.4)	0.22 (.06-.57)	3.4 (.9-8.9)	6.3 (1.6-16)
3	3.1 (.8-8.1)	2.0 (.52-5.2)	0.33 (.09-.86)	1.1 (.29-2.9)	1.6 (.42-4.2)
4	32 (8.3-83)	2.0 (.5-5.2)	0.7 (.2-2.0)	2.2 (.6-5.7)	2.2 (.6-5.7)
5	53 (14-140)	6.3 (1.6-16)	1.4 (.36-3.6)	1.4 (.36-3.6)	1.4 (.36-3.6)
7	140 (36-360)	45 (12-120)	4.4 (1.1-11)	9.9 (2.5-25)	6.5 (1.7-17)
9	220 (57-570)	22 (5.7-57)	56 (15-150)	7.3 (1.9-19)	8.6 (2.2-22)
15	--	530 (140-1400)	180 (47-470)	87 (23-230)	33 (8.6-86)
17	--	730 (190-1900)	310 (81-810)	150 (39-390)	520 (130-1300)
22	--	--	330 (86-860)	1500 (390-3900)	570 (150-1500)

Table 20

EFFECT OF DDS ON CFU

The CFU (x 10^{-6}) is shown for each drug concentration at each observation period. The observation period is shown in the first column. The concentration of cells that formed colonies is shown in a column for each drug concentration. The ± 95% confidence limits are shown in parentheses.

Day	DDS in μg/ml				
	0	0.3	1.0	3.0	10.0
0	0.7 (.35-1.05)	0.65 (.3-1.0)	0.55 (2.-29)	0.4 (.1-.7)	0.5 (.2-.8)
3	1.2 (.2-2.2)	0.6 (.35-.85)	0.7 (.35-1.1)	0.5 (.4-.6)	1.1 (.65-1.6)
9	600 (100-1100)	70 (20-120)	21 (17-25)	18 (15-21)	4.7 (3.8-5.6)
17	--	720 (637-800)	197 (144-250)	175 (120-230)	276 (170-380)
22	--	--	350 (250-450)	870 (770-970)	390 (320-460)

Table 21

EFFECT OF DDS ON TURBIDITY AND TOTAL NUMBERS

The measurement of turbidity in Klett units is shown for each observation period. The Klett units were transformed to total cells/ml (x 10^{-6}) which are shown in parentheses. The observation period is shown in the first column. The turbidity and cellular concentration are shown in a column for each drug concentration.

Day			DDS in µg/ml		
	0	0.3	1.0	3.0	10.0
0	0	0	0	0	0
1	1	0	0	0	0
2	6	0	0	0	0
3	16	4	2	0	4
4	26(20)	9	4	1	6
5	49(33)	17	9	6	11
6	85(56)	27(21)	12	8	10
7	108(67)	28(21)	15	8	9
8	171(119)	40(27)	21(18)	10	13
9	261	48(33)	24(19)	15	16
10	--	66(44)	37(25)	21(18)	18
15	--	216(142)	91(59)	50(34)	32(23)
17	--	345	123(76)	71(47)	45(30)
22	--	—	195(131)	148(100)	94(60)

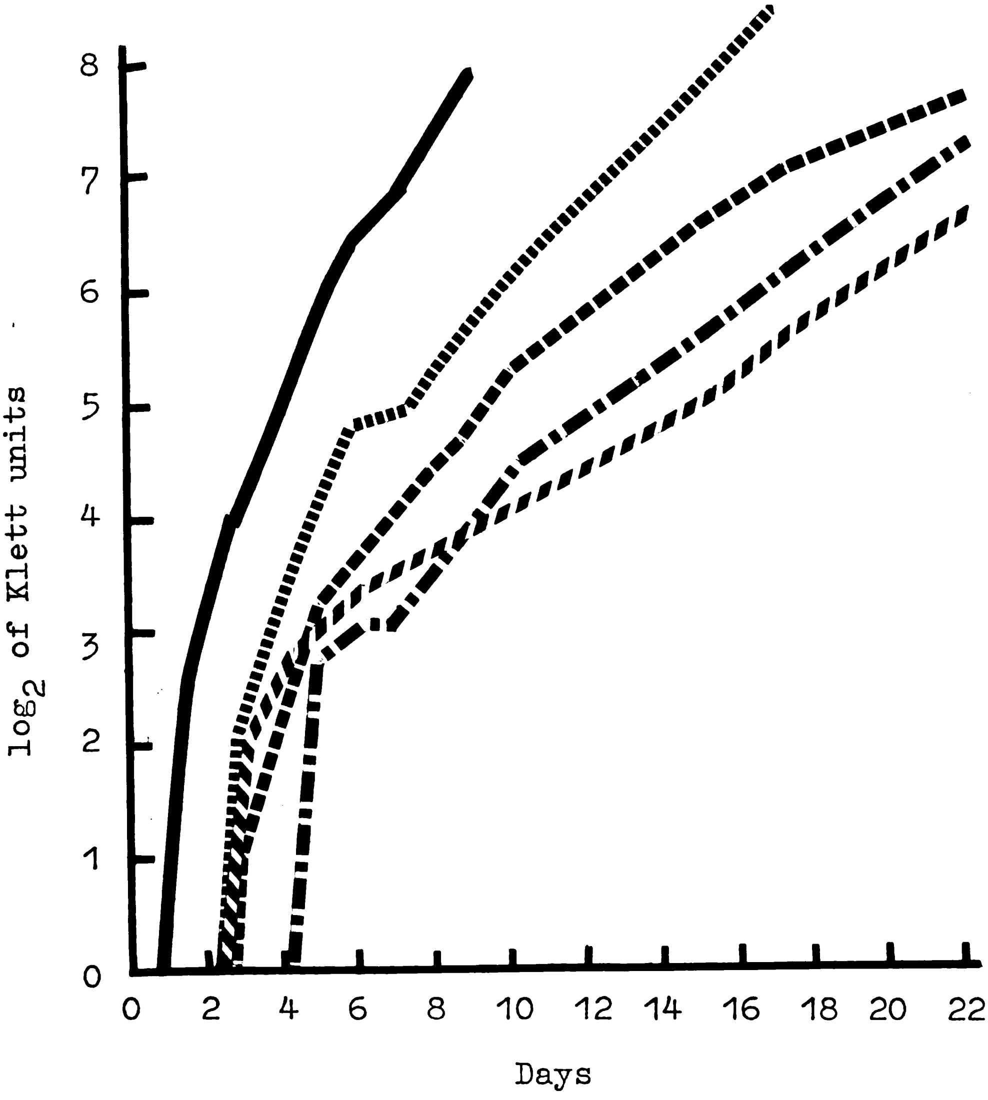
Cellular concentration can not be estimated from Klett units of less than 20 nor more than 250.

Figure 2

EFFECT OF DDS ON TURBIDITY

Growth in Klett units is shown in logarithmic scale on the ordinate. Time in days is shown on the abscissa.

(━━━) -- 0 DDS; (┅┅┅) -- 0.3 μg/ml; (▬▬▬) -- 1.0 μg/ml; (▬▪▬) -- 3.0 μg/ml; (////) -- 10 μg/ml.



measurements, it was known that one cell of M. kansasii can multiply sufficiently to produce turbidity in 22 days. Therefore cultures that contained drug but did not produce turbidity until day 22 were interpreted as probably containing resistant organisms. These were tested for resistance by plating on Dubos agar containing DDS. Cultures that did not produce turbidity by 24 days could either be the result of bactericidal action by DDS, or they could contain resistant mutants for which the growth rate was much slower than that among uninhibited populations. Therefore, four tubes that contained 100 μg/ml DDS and 1.8×10^4 cells per ml were filtered aseptically using 0.15 μ average pore-diameter membrane filters. The filters with the retained bacilli were washed by passage of sterile Dubos broth, and then transferred to Dubos agar and incubated. Parallel filters were inoculated with M. kansasii that had not been exposed to DDS and incubated. The filters were observed twice weekly for six weeks.

The number of days before the appearance of turbidity is shown in Table 22. Among cultures containing the largest inoculum, which were already turbid, the condition observed on day 1 was a very large increase in turbidity. Qualitatively, 1.8×10^6 cells/ml were not inhibited by any concentration of DDS within the 2 days necessary for the development of turbidity in those cultures. Cultures containing 1.8×10^5 and 1.8×10^4 cells/ml were inhibited by 0.1 μg/ml DDS, but apparently only by a slowing of the growth rate of the entire population. Ten ml of broth containing 1.8×10^5 cells/ml or a total population of 1.8×10^6 cells appears to contain mutants

Table 22

EFFECT OF INOCULUM SIZE ON INHIBITION BY DDS IN BROTH

Response is shown as the number of days to the appearance of turbidity. The inoculum size is shown in the first column as the the number of cells/ml initially present in the cultures. The results are shown in a column for each concentration of DDS. -- indicates no turbidity.

Inoculum	DDS in μg/ml				
cells/ml	0	0.1	1.0	10	100
1.8×10^7	1	1	1	1	1
1.8×10^6	2	2	2	2	2
1.8×10^5	4	6	22	22	--
1.8×10^4	7	8	22	--	--

resistant to 1 or 10 µg/ml DDS. Mutants resistant to 1 µg/ml DDS appeared regularly in ten populations of 1.8×10^5 cells. In three sets of tubes, turbidity failed to develop. Among the cells filtered from the four tubes mentioned above, no colonies developed, although the control filters showed growth by two and a half weeks. Therefore, 100 µg/ml DDS was totally bactericidal to M. kansasii in this inoculum size.

THE EFFECT OF LOW LEVELS OF DDS ON M. KANSASII IN STATIONARY CULTURE

Seven replicate sets of tubes were prepared with DDS present in concentrations of 0, 0.05, 0.1, 0.2, 0.4, and 0.8 µg/ml in Dubos broth. The final inoculated cultures contained 6.7×10^5 cells/ml. The cultures were incubated at 37°C. The O.D. was measured three times weekly.

The results of this experiment are shown in Tables 23-25 and Figures 3 and 4. Table 23 shows the O.D. measurements for each observation period. Figure 3 is a graph of growth versus time. Table 24 shows the per cent inhibition produced by each drug concentration at each observation period. Figure 4 is a graph of per cent inhibition versus concentration of DDS. Table 25 shows estimates of the doubling time of M. kansasii in the presence of low levels of DDS.

The tabulated O.D. measurements, Table 23, show that there is significant inhibitory effect of DDS in this concentration range. The growth curves in Figure 3 show in addition that the inhibitory effect is not expressed until after the fourth day of exposure to

Table 23

EFFECT OF LOW LEVELS OF DDS ON O.D.

The mean O.D. x 10^3 is shown for each observation period. The observation period is shown in the first column. The mean O.D. is shown in a column for each concentration of drug. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml					
	0	0.05	0.1	0.2	0.4	0.8
2	13(2)	10(1)	13(1)	12(3)	14(2)	14(2)
4	35(3)	30(2)	32(2)	32(3)	33(3)	31(3)
7	57(3)	43(5)*	47(3)*	45(5)	46(5)	43(4)*
9	82(3)	51(7)*	56(2)*	47(3)*	47(4)*	45(4)*
11	124(4)	63(9)*	71(3)*	61(5)*	57(5)*	50(3)*
14	254(7)	83(17)*	101(3)*	85(6)*	74(3)*	61(4)*
16	345(10)	101(19)*	118(5)*	102(5)*	83(6)*	71(2)*
18	460(9)	117(29)*	156(11)*	128(6)*	104(7)*	77(3)*
21	642(22)	183(33)*	208(20)*	167(9)*	142(6)*	99(9)*
23	790(37)	209(53)*	255(29)*	209(17)*	170(10)*	119(8)*
25	920(40)	250(84)*	296(37)*	231(24)*	210(15)*	145(11)*

* Significant at the $P < .001$ level compared to control.

Figure 3

EFFECT OF LOW LEVELS OF DDS ON O.D.

Growth in O.D. units x 10^3 is shown in logarithmic scale on the ordinate. Time in days is shown on the abscissa. (▬▬) -- 0 DDS; (⁄⁄⁄) -- 0.05 µg/ml; (┃┃┃┃┃) -- 0.1 µg/ml; (▬▬▪) -- 0.2 µg/ml; (▪▪▪▪) -- 0.4 µg/ml; (▬▪▬▪) -- 0.8 µg/ml.

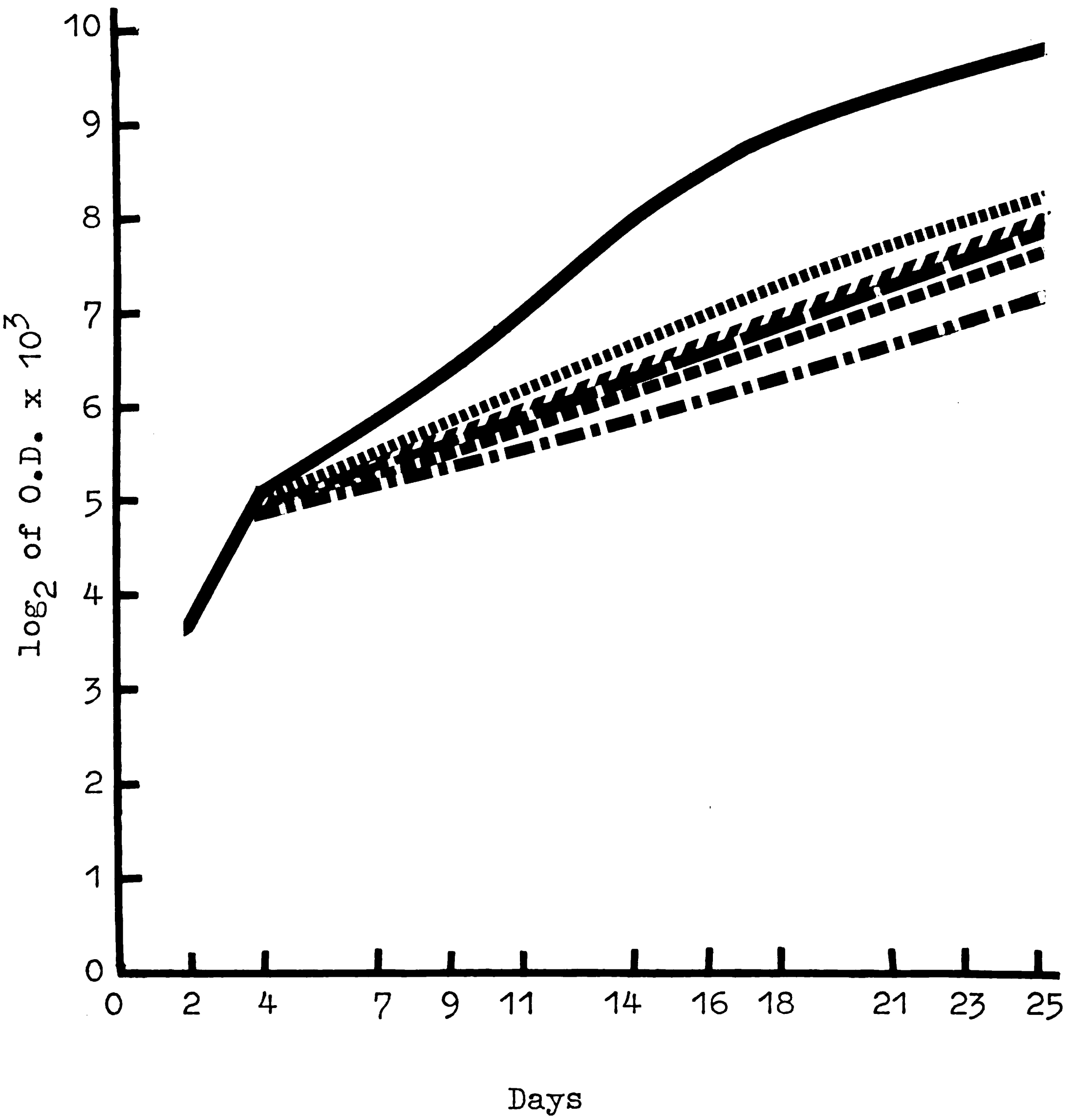


Table 24

PER CENT INHIBITION PRODUCED BY LOW LEVELS OF DDS

The per cent inhibition is shown for each drug concentration at each observation period. The observation period is shown in the first column. The per cent inhibition is shown in a column for each concentration of drug. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml				
	0.05	0.1	0.2	0.4	0.8
2	0 (0-40)	0 (0-20)	0 (0-40)	0 (0-20)	0 (0-20)
4	14.3 (0-26)	8.6 (0-21)	8.6 (0-29)	5.7 (0-21)	11.4 (0-26)
7	24.6 (11-37)	17.5 (7-27)	21.1 (7-33)	19.3 (5-33)	24.6 (2-35)
9	37.8 (27-48)	31.7 (27-37)	42.7 (37-48)	42.7 (35-49)	45.1 (38-52)
11	49.2 (40-58)	42.7 (38-47)	50.8 (45-56)	54.0 (48-59)	59.7 (56-63)
14	67.3 (59-75)	60.2 (58-63)	66.5 (63-70)	70.9 (69-73)	76.0 (74-78)
16	70.7 (64-77)	65.8 (63-68)	70.4 (68-73)	75.9 (73-78)	79.4 (78-81)
18	74.4 (68-81)	66.1 (63-69)	72.1 (70-74)	77.4 (75-79)	83.3 (82-84)
21	71.5 (65-77)	67.6 (57-72)	74.0 (71-76)	77.9 (76-80)	84.6 (82-86)
23	73.5 (65-81)	67.7 (62-73)	73.5 (70-77)	78.5 (76-81)	84.9 (83-87)
25	72.8 (62-83)	67.8 (62-73)	74.9 (71-78)	77.2 (74-80)	84.2 (82-86)

Figure 4

PER CENT INHIBITION PRODUCED BY LOW LEVELS OF DDS

Per cent inhibition is plotted on the ordinate. The $\log_{10}$ concentration of DDS is plotted on the abscissa. (▬▪▬▪) -- day 7; (▪▬▬) -- day 9; (▰▰▰▰) -- day 11; (▮▮▮▮▮▮▮) -- day 14; (■■■■) -- day 25. The vertical bars are the 95% confidence range.

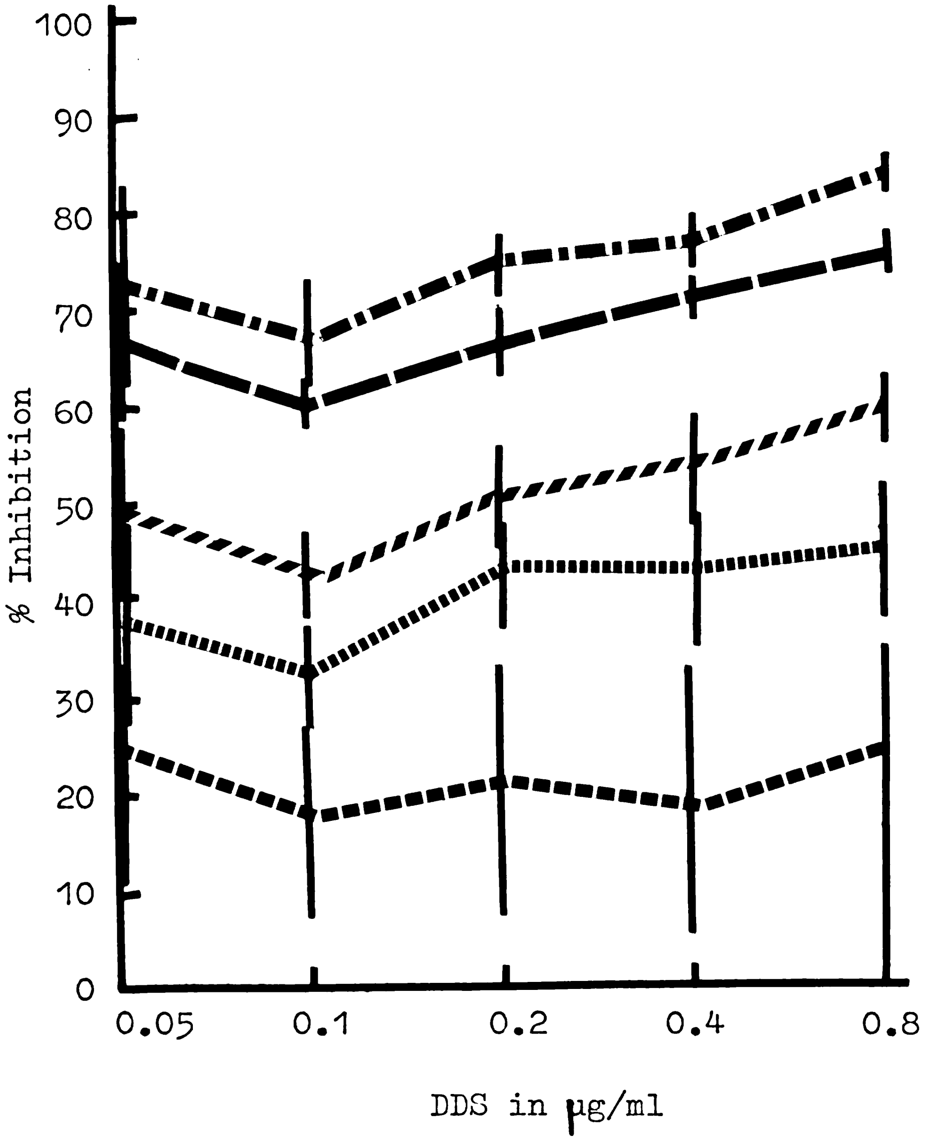


Table 25

GROWTH RATES OF M. KANSASII INHIBITED BY DDS

The rate of growth was estimated as the time interval in days for a doubling of the O.D. and was derived from the curves in Figure 3. The interval during which the given growth rates were observed is shown in the first column. The doubling times are shown in a column for each drug concentration.

Days	DDS in µg/ml					
	0	0.05	0.1	0.2	0.4	0.8
2-4	1.6	1.6	1.6	1.6	1.6	1.6
4-9	3.9	7.0	7.1	8.7	8.7	9.8
9-14	3.1	7.0	7.1	6.6	7.4	11.8
14-18	4.5	7.0	7.1	6.6	7.4	11.8
18-25	6.8	7.0	7.1	6.6	7.4	7.9

DDS. The inhibition is clearly a dose-related decrease in the growth rate starting at 0.1 µg/ml.

The apparently greater inhibition of the cultures exposed to 0.05 µg/ml as compared to the 0.1 µg/ml concentration has resulted from the irregularity of the results for the lower concentration. The difference between the growth in the presence of 0.05 µg/ml DDS and that in the presence of 0.1 µg/ml is not statistically significant. The growth in cultures containing 0.2 µg/ml was more inhibited than in cultures containing half that concentration, beginning on day 9. Beginning on day 14, the cultures exposed to 0.4 µg/ml were more inhibited than those containing 0.2 µg/ml. Beginning on day 9, the cultures exposed to 0.4 µg/ml showed greater inhibition than the 0.1 µg/ml cultures. The highest concentration, 0.8 µg/ml, exerted greater inhibition than did 0.4 µg/ml from day 14; inhibition was greater in the 0.8 µg/ml cultures than in the 0.2 µg/ml cultures from day 11 and than the 0.1 µg/ml cultures from day 9.

The pattern is one of gradually increasing inhibition over a two week period followed by a rather constant level of inhibition. At this constant level, none of these low concentrations produced less than 50% inhibition, nor did the 95% confidence interval approach 50% inhibition. Because none of the drug concentrations used here produced less than 50% inhibition it is not possible to find the IC50 by interpolation.

In this experiment there was an initial period of normal growth before the onset of inhibition. This is in direct contrast to the apparent prolongation of the lag phase seen in the previous

experiment. However, the cultures were stationary in this experiment, whereas they were shaken in the previous one.

THE EFFECT OF PABA ON M. KANSASII IN STATIONARY CULTURE

Seven replicate sets of tubes were prepared with PABA present in concentrations of 0, 0.01, 0.1, 1.0, and 10.0 µg/ml in Dubos broth. The final inoculated cultures contained 6.7×10^5 cells/ml. The cultures were incubated at 37°C. The O.D. was measured three times weekly.

The results of this experiment are shown in Tables 26 and 27 and in Figure 5. Table 26 shows the O.D. measurements for each observation period. Figure 5 is a graph of growth versus time. Table 27 shows the per cent inhibition produced by each PABA concentration at each observation period.

The striking result is the inhibition of M. kansasii by 10 µg/ml PABA. The inhibition developed during two weeks of incubation to a level of about 30% which was maintained for the remainder of the experiment. This is the same pattern produced by DDS, namely one of gradually increasing inhibition over a two week period which then becomes quite constant.

It seems probable that the inhibitory effect of PABA at 10 µg/ml will continue to be expressed in the presence of DDS. If so, this concentration would mark the upper level of PABA that can be used to counteract DDS inhibition in experiments in which this would be desirable. The following experiment explores this possibility.

Table 26

EFFECT OF PABA ON O.D.

The mean O.D. x 10^3 is shown for each observation period. The observation period is shown in the first column. The mean O.D. is shown in a column for each concentration of PABA. The ± 95% confidence limits are shown in parentheses.

Day	PABA in µg/ml				
	0	0.01	0.1	1.0	10.0
2	13(2)	12(3)	9(4)	12(3)	14(3)
4	35(3)	32(5)	30(1)	31(3)	30(1)
7	57(3)	57(4)	54(3)	56(4)	51(2)
9	82(3)	87(6)	79(7)	84(6)	74(2)*
11	124(4)	130(13)	132(13)	136(14)	106(6)*
14	254(7)	257(26)	273(30)	273(19)	182(6)*
16	345(10)	350(33)	365(32)	363(32)	238(11)*
18	460(9)	467(42)	477(45)	483(38)	326(10)*
21	642(22)	642(59)	679(60)	680(45)	448(20)*
23	790(37)	773(66)	821(75)	832(42)	566(12)*
25	920(40)	870(90)	1000(110)	1000(60)	694(19)*

* Significant at the $P < .001$ level compared to control.

Figure 5

EFFECT OF PABA ON O.D.

Growth in O.D. unite x 10^3 is shown in logarithmic scale on the ordinate. Time in days is shown on the abscissa. (▬) -- 0 PABA; (▮▮▮▮▮) -- 10 µg/ml. The data for 1.0, 0.1, and 0.01 µg/ml PABA are essentially identical to that for 0 PABA.

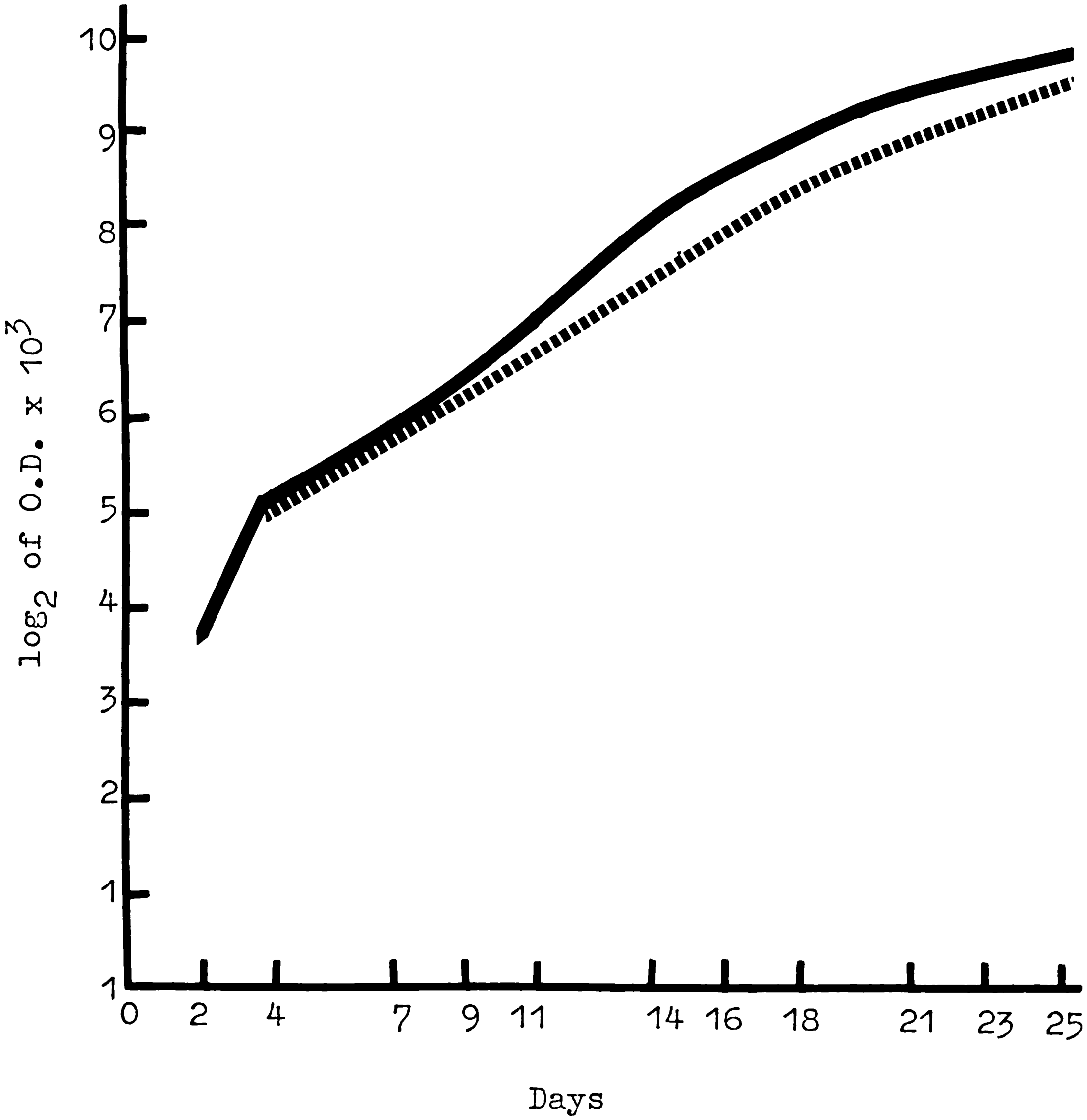


Table 27

PER CENT INHIBITION PRODUCED BY PABA

The per cent inhibition is shown for each PABA concentration at each observation period. The observation period is shown in the first column. The per cent inhibition is shown in a column for each concentration of PABA. The ± 95% confidence limits are shown in parentheses.

Day	PABA in µg/ml			
	0.01	0.1	1.0	10.0
2	7.7 (0-40)	30.8 (0-66)	7.7 (0-40)	0 (0-27)
4	8.6 (0-29)	14.3 (3-29)	11.4 (0-26)	14.3 (3-24)
7	0 (0-12)	5.3 (0-15)	1.8 (0-13)	10.5 (2-18)
9	0 (0-5)	3.7 (0-15)	0 (0-8)	9.8 (4-15)
11	0 (0-9)	0 (0-7)	0 (0-5)	14.5 (7-22)
14	0 (0-11)	0 (0-8)	0 (0-3)	28.3 (24-33)
16	0 (0-11)	0 (0-6)	0 (0-7)	31.0 (26-36)
18	0 (0-9)	0 (0-8)	0 (0-5)	29.1 (25-33)
21	0 (0-12)	0 (0-7)	0 (0-4)	30.2 (25-36)
23	2.2 (0-15)	0 (0-10)	0 (0-4)	28.4 (23-33)
25	5.4 (0-19)	0 (0-7)	0 (0-2)	24.6 (19-30)

REVERSAL BY PABA OF THE INHIBITION OF M. KANSASII BY DDS

Seven replicate sets of tubes were prepared with Dubos broth. DDS was present at concentrations of 0 and 0.8 μg/ml. In each concentration of DDS, PABA was present in concentrations of 0, 0.01, 0.1, 1.0, and 10.0 μg/ml. In a final set, DDS was present at a concentration of 0.1 μg/ml alone or with 0.1 μg/ml PABA. The final inoculated cultures contained 6.7 x 10^5 cells/ml. The cultures were incubated at 37°C. The O.D. was measured three times a week.

The results of this experiment are presented in Tables 28-32 and Figure 6. The O.D. measurements are shown in Table 28 for DDS at 0.8 μg/ml and Table 29 for DDS at 0.1 μg/ml. Figure 6 is a graph of growth versus time. Tables 30 and 31 show per cent inhibition for the high and low concentrations of DDS respectively. Table 32 shows the per cent reversal of DDS inhibition by PABA. For this calculation, the difference between the O.D. of the cultures containing DDS alone and the cultures containing no drug is taken as 100% inhibition, and 0% reversal of inhibition. This number is then divided by the difference in O.D. between the cultures containing DDS alone and that containing DDS and PABA.

One μg/ml of PABA reversed the inhibition of 0.8 μg/ml DDS completely. Ten μg/ml PABA itself produced inhibition which was not significantly increased nor decreased by the presence of 0.8 μg/ml DDS. Concentrations of PABA smaller than 1 μg/ml partially antagonized 0.8 μg/ml DDS. PABA, 0.1 μg/ml, reversed inhibition by 0.8 μg/ml DDS by about 67%. PABA, 0.01 μg/ml reversed DDS inhibition by only about 12%, this value is statistically significant.

Table 28

EFFECT OF DDS AND PABA ON O.D., PART I

The mean O.D. x 10^3 is shown for each observation period. The observation period is shown in the first column. The mean O.D. is shown in a column for each concentration of drug. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml					
	0	0.8	0.8	0.8	0.8	0.8
	PABA in µg/ml					
	0	0	0.01	0.1	1.0	10.0
2	13(2)	14(2)	17(7)	13(2)	15(3)	13(7)
4	35(3)	31(3)	37(8)	34(2)	33(4)	34(5)
7	57(3)	43(4)*	48(8)	48(6)	58(3)	57(6)
9	82(3)	45(4)*	56(10)*	64(4)*	93(8)	83(5)
11	124(4)	50(3)*	61(13)*	85(8)*	134(8)	112(4)*
14	254(7)	61(4)*	88(20)*	159(27)*	259(29)	196(20)*
16	345(10)	71(2)*	103(32)*	225(33)*	344(39)	261(15)*
18	460)9)	77(3)*	121(28)*	334(34)*	450(39)	335(23)*
21	642(22)	99(9)*	161(39)*	445(36)*	637(45)	448(33)*
23	790(37)	119(8)*	196(51)*	551(14)*	772(39)	550(32)*
25	920(40)	145(11)*	258(43)*	690(54)*	909(69)	651(33)*

* Significant at the $P < .001$ level compared to control

Table 29

EFFECT OF DDS AND PABA ON O.D., PART II

The mean O.D. x 10^3 is shown for each observation period. The observation period is shown in the first column. The mean O.D. is shown in a column for each concentration of drug. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml			
	0	0.1	0.1	0
	PABA in µg/ml			
	0	0	0.1	0.1
2	13(2)	13(1)	18(7)	9(4)
4	35(3)	32(2)	35(4)	30(1)
7	57(3)	47(3)*	62(4)	54(3)
9	82(3)	56(2)*	95(6)	79(7)
11	124(4)	71(3)*	145(9)	132(13)
14	254(7)	101(3)*	290(35)	273(30)
16	345(10)	118(5)*	396(57)	365(32)
18	460(9)	156(11)*	502(66)	477(45)
21	642(22)	208(20)*	670(51)	679(60)
23	790(37)	255(29)*	795(80)	821(75)
25	920(40)	296(37)*	920(60)	1000(110)

* Significant at the P<.001 level compared to control.

Figure 6

EFFECT OF DDS AND PABA ON O.D.

Growth in O.D. x 10^3 is shown in logarithmic scale on the ordinate. Time in days is shown on the abscissa. (━━━) -- 0 PABA, 0 DDS; (▬ ━━) -- 0.1 µg/ml PABA + 0.8 µg/ml DDS; (▪▪▪▪▪▪) -- 0.01 µg/ml PABA + 0.8 µg/ml DDS; (▬▪▬▪) -- 0 PABA + 0.8 µg/ml DDS. PABA 1.0 µg/ml + 0.8 µg/ml DDS is identical to (━━━). PABA 10.0 µg/ml + 0.8 µg/ml DDS is similar to (━━ ▬).

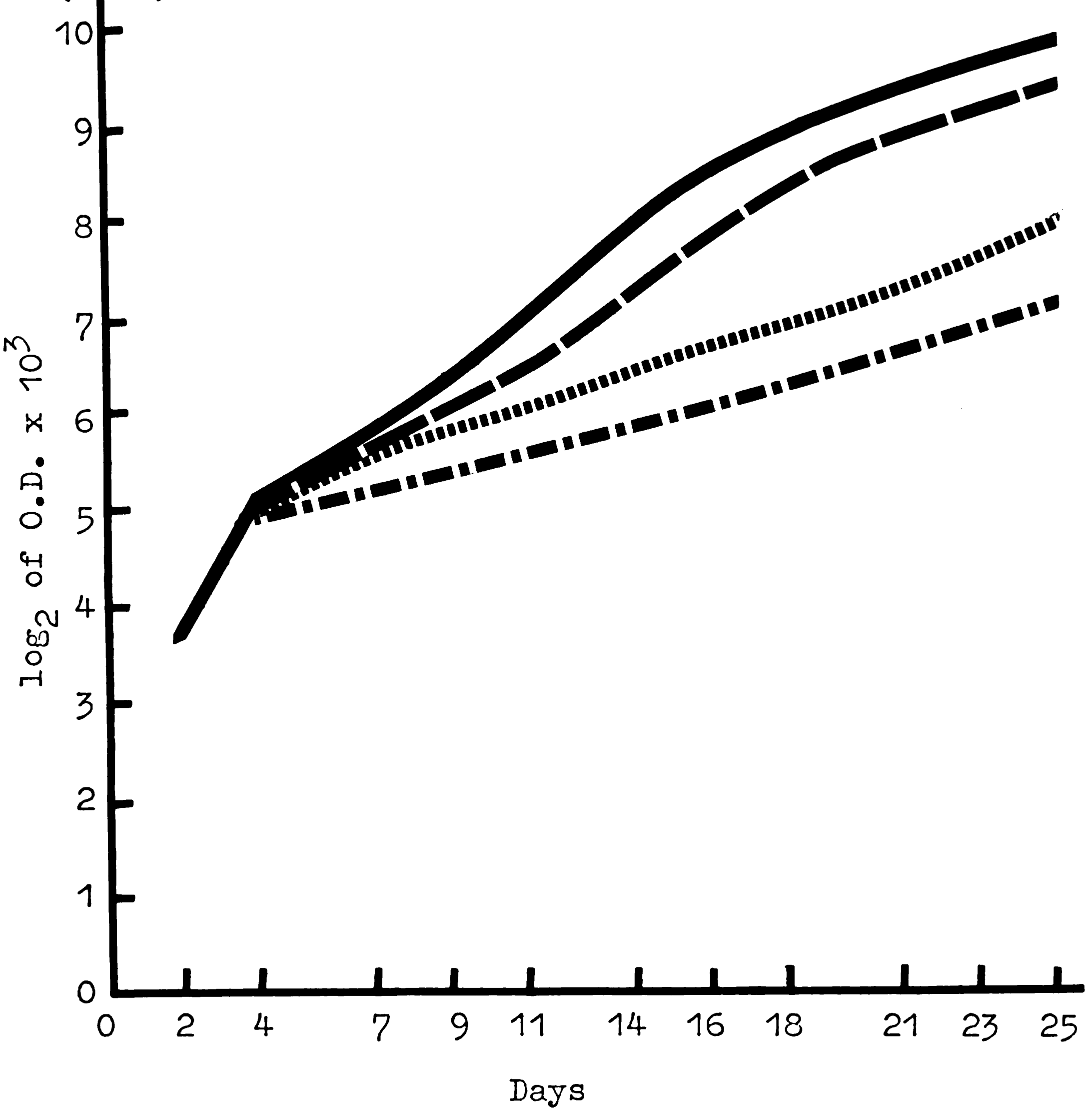


Table 30

PER CENT INHIBITION WITH DDS AND PABA, PART I

The per cent inhibition is shown. The observation period is shown in the first column. The per cent inhibition is shown in a column for each concentration of drug. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml				
	0.8	0.8	0.8	0.8	0.8
	PABA in µg/ml				
	0	0.01	0.1	1.0	10.0
2	0 (0-20)	0 (0-33)	0 (0-27)	0 (0-20)	0 (0-60)
4	11.4 (0-26)	0 (0-24)	2.9 (0-16)	5.7 (0-24)	2.9 (0-24)
7	24.6 (12-35)	15.8 (0-33)	15.8 (0-30)	0 (0-8)	0 (0-15)
9	45.1 (38-52)	31.1 (16-46)	23.2 (15-31)	0 (0-0)	0 (0-8)
11	59.7 (56-63)	50.8 (33-63)	31.5 (22-40)	0 (0-2)	9.7 (3-16)
14	76.0 (74-78)	65.4 (56-74)	37.4 (27-47)	0 (0-12)	22.9 (12-33)
16	79.4 (78-81)	70.1 (60-80)	34.8 (23-46)	0.3 (0-14)	24.3 (18-31)
18	83.3 (82-84)	73.7 (67-80)	27.4 (18-36)	2.2 (0-12)	27.2 (20-34)
21	84.6 (83-86)	74.9 (67-82)	30.7 (22-38)	0.8 (0-11)	30.2 (22-38)
23	84.9 (83-87)	75.2 (67-82)	30.3 (25-35)	2.3 (0-11)	30.4 (23-36)
25	84.2 (82-86)	72.0 (66-78)	25.0 (15-34)	1.2 (0-13)	29.2 (22-36)

Table 31

PER CENT INHIBITION WITH DDS AND PABA, PART II

The observation period is shown in the first column. The per cent inhibition is shown in a column for each combination of drug concentrations. The ± 95% confidence limits are shown in parentheses.

Day		**DDS in µg/ml**	
	0.1	0.1	0
		PABA in µg/ml	
	0	0.1	0.1
2	0 (0-20)	0 (0-27)	30.8 (0-66)
4	8.6 (0-21)	0 (0-18)	14.3 (3-29)
7	17.5 (7-27)	0 (0-3)	5.3 (0-15)
9	31.7 (27-37)	0 (0-0)	3.7 (0-15)
11	42.7 (38-47)	0 (0-0)	0 (0-7)
14	60.2 (58-63)	0 (0-3)	0 (0-8)
16	65.8 (63-68)	0 (0-5)	0 (0-6)
18	66.1 (63-69)	0 (0-7)	0 (0-8)
21	67.6 (37-72)	0 (0-7)	0 (0-7)
23	67.7 (62-73)	0 (0-14)	0 (0-10)
25	67.8 (62-73)	0 (0-10)	0 (0-7)

Table 32

PER CENT REVERSAL OF DDS INHIBITION BY PABA

The per cent reversal of DDS inhibition by PABA is shown. The method of calculation is given on page 30. The observation period is shown in the first column. The per cent reversal of inhibition is shown in a column for each concentration of PABA.

Day	DDS in µg/ml			
	0.8	0.8	0.8	0.8
	PABA in µg/ml			
	0.01	0.1	1.0	10.0
2	-	-	-	-
4	-	-	-	-
7	35.7	35.6	100	100
9	29.7	48.6	100	100
11	14.9	47.3	100	83.8
14	14.0	50.8	100	69.9
16	11.7	56.2	99.6	69.3
18	11.5	67.1	97.4	67.4
21	11.4	67.0	99.1	64.3
23	11.5	64.4	97.3	64.2
25	13.7	66.1	92.6	61.3

Growth in the presence of 0.1 µg/ml PABA is not significantly different from that in the control, and is the same in the presence of 0.1 µg/ml DDS, even though this concentration of DDS alone produces about 67% inhibition.

The presence of PABA does not delay the expression of DDS inhibition in partially effective concentrations. Inhibition is still apparent starting from day 4, and the rate of growth is merely slowed less by DDS when PABA is also present. Inhibition develops to a constant level over two weeks just as in the absence of PABA.

It should be noted that in Table 32, the per cent reversal recorded in the columns headed 0.01 and 0.1 µg/ml PABA was based on O.D. measurements significantly different from those of the control cultures, from those containing DDS alone, and from those containing PABA alone. The per cent reversal recorded in the columns headed 1.0 and 10.0 µg/ml PABA was based on O.D. measurements which were not significantly different from those of the cultures containing PABA alone.

REVERSAL OF DDS INHIBITION BY PABA USING THE RESAZURIN METHOD

Resazurin broth was prepared in tubes to contain the following drug concentrations: DDS 0 and 10 µg/ml, and PABA 0, 0.01, 0.1, 1.0, and 10.0 µg/ml for a total of 10 combinations. Two inocula were used: inoculum A was a barely turbid suspension of M. kansasii, and Inoculum B was a 1:10 dilution of inoculum A.

The results from this experiment are shown in Table 33. The results were identical for the two inocula. M. kansasii was inhibited

Table 33

REVERSAL OF DDS INHIBITION BY PABA USING RESAZURIN

The response is given for combinations of DDS and PABA. G represents growth, and in the presence of drug it also represents resistance to that concentration of drug. I represents inhibition or susceptibility to that concentration of drug. DDS concentrations are given in the first column. The responses are shown in a column for each concentration of PABA.

DDS in ug/ml	PABA in µg/ml				
	0	0.01	0.1	1.0	10.0
0	G	G	G	G	I
10.0	I	I	I	G	I

by 10 μg/ml PABA, and by 10 μg/ml DDS. PABA 1 μg/ml reversed the inhibition produced by 10 μg/ml DDS, whereas 0.1 μg/ml PABA was not sufficient to reverse the inhibition produced by the DDS. However, neither PABA nor DDS was able to reverse the inhibitory action of the other when both were present at the concentration of 10 μg/ml.

EFFECT OF VARYING DURATION OF DRUG CONTACT IN BROTH ON COLONY FORMATION BY M. KANSASII

Quadruplicate tubes were prepared containing 10 ml Dubos broth with the following concentrations of DDS: 1, 2, 4, and 8 μg/ml. A turbid suspension of M. kansasii was used; the concentration of bacilli was estimated by O.D. measurement. The suspension was diluted with sterile broth to yield a cellular concentration of between 1×10^5 and 5×10^5 CFU/ml. The drug-containing tubes were inoculated with 0.01, 0.03, 0.1, and 0.3 ml of this suspension and incubated at 37°C.

Following one, four and eight days of incubation, 0.1 ml aliquots were removed from each of the tubes, and spread on Dubos agar plates, which were then incubated at 37°C. Four plates were inoculated for each concentration of DDS. Colonies were counted weekly from the third week through the eighth week of the experiment.

The results of this experiment are presented in Tables 34 and 35. The total number of colonies for four plates is recorded in Table 34 and the per cent reduction in CFU during the various intervals is shown in Table 35.

Table 34

SURVIVAL OF *M. KANSASII* IN BROTH CONTAINING DDS

The number of colonies on four plates is shown for each drug concentration and each duration of drug contact. The first column shows the duration of drug contact in days. A column for each concentration of DDS shows the number of colonies. The ± 95% confidence limits are shown in parentheses.

Duration of drug contact	DDS in µg/ml 1	2	4	8
1	690(51)	933(60)	674(51)	761(54)
4	296(34)	357(37)	220(50)	58(15)
8	121(11)	32(11)	0	0

Table 35

PER CENT REDUCTION IN COLONIES

The difference in numbers of colonies between any two observation periods (Table 34) is expressed here as per cent reduction in colony forming units. The interval is shown in the first column. The per cent reduction in colonies is shown in a column for each drug concentration. The ± 95% confidence limits are shown in parentheses.

Interval in days	DDS in µg/ml			
	1	2	4	8
1-4	57(47-65)	62(55-68)	67(55-77)	92(90-95)
1-8	82(78-88)	97(95-98)	100	100
4-8	59(46-70)	91(87-95)	100	100

The χ^2 values for the various pairs of colony counts show that the values are significantly different ($P < 0.005$) with the following four exceptions. After one day of incubation, the number of colonies on the plates prepared from the cultures containing 1 μg/ml DDS was not significantly different ($P > 0.05$) from the numbers of colonies on the plates streaked from the cultures containing 4 or 8 μg/ml DDS. The numbers of colonies on the plates prepared after one day's incubation from the cultures containing 4 and 8 μg/ml were different at the $P = 0.025$ level, as were the numbers of colonies on the plates prepared after four days' incubation from the cultures containing 1 and 2 μg/ml DDS.

It is apparent that the higher drug concentrations exerted a greater effect on colony formation only after more than one day of contact. A bactericidal effect has obviously resulted from exposure of the organisms to all drug concentrations for four or eight days.

The null hypothesis that the median number of colonies is identical for each drug concentration was tested and accepted at the $P = 0.05$ level. The null hypothesis that the median number of colonies is identical for each duration of drug contact was tested and rejected at the $P = 0.025$ level in favor of the hypothesis that the duration of contact influenced the number of colonies formed.

The rate of colony appearance was analysed as a function of both drug concentration and duration of exposure to drug. After one day of drug contact, the bacilli exposed to 2 μg/ml formed colonies significantly more slowly than did those exposed to 1 μg/ml. Similarly, the organisms exposed to 8 μg/ml formed colonies more slowly

than did those from lower drug concentrations plated on the same day. Obviously, the eight-day samples from which no colonies formed -- from the cultures containing 4 and 8 µg/ml -- represent the extreme in slowed colony formation. The remaining comparisons among the organisms plated from the various drug concentrations did not demonstrate differences. In some cases in which differences were anticipated, the number of colonies was small, generating a very wide range within the ± 95% confidence limits, and thus perhaps obscuring the expected differences. For example, the colonies from the culture plated after exposure for four days to 1 µg/ml DDS appeared more slowly than those from the organisms exposed for only one day to the same drug concentration. However, the number of colonies appearing on the plates prepared from the culture exposed for eight days had such a broad 95% confidence band that no conclusions can be drawn.

DDS SUSCEPTIBILITY OF <u>M. KANSASII</u> STRAINS THAT GREW IN MEDIUM CONTAINING DDS

Colonies that grew on drug-containing agar plates were picked up with a flamed inoculating needle and propagated on Dubos agar slants. Broth cultures that became turbid in the presence of DDS were inoculated onto Dubos agar plates. All strains were tested by a series of taxonomic tests including acid-fast staining, colony morphology on Dubos agar, three-day and two-week aryl sulfatase, nitrate reduction and Tween hydrolysis, to determine whether they exhibited the characteristics of the parent strain. Thirty-six

strains which were identical to the parent strain by all these tests were tested for DDS susceptibility using the resazurin method.

The results of this experiment are shown in Table 36. Six of seven strains tested with DDS concentrations of 1, 2, 3, 5, and 10 µg/ml were susceptible to all these concentrations. Three of these strains had grown on agar containing 0.1 µg/ml DDS; one strain each had grown on agar containing 1, 2, or 3 µg/ml. One strain isolated from agar containing 0.1 µg/ml DDS was found to be resistant to 2 µg/ml but susceptible to 3 µg/ml.

Of 29 strains tested on DDS concentrations of 0.3, 1, 3, and 10 µg/ml DDS, 25 were susceptible to all the tested concentrations. Seventeen had been isolated from media containing a higher concentration of DDS than that to which they were shown susceptible, one was susceptible to the same concentration from which it had been isolated, and seven had been isolated from media containing concentrations of DDS lower than those used in this test. The remaining four strains were resistant to 0.3 µg/ml DDS but susceptible to 1 µg/ml. Three had been isolated from agar containing 10 µg/ml DDS and the other one was isolated from a broth culture that had contained 2 µg/ml DDS.

Of all the tested strains, 26 were derived from experiments in which all colonies suspected of representing resistant mutants were cultured. The total number of CFU plated on DDS-containing agar in these experiments was 95,300. That is, 1 in 3,700 CFU actually formed colonies on agar containing DDS. Of these 26 strains, however, only four demonstrated reduced susceptibility to DDS compared to the

Table 36

SUSCEPTIBILITY OF STRAINS THAT GREW ON DDS MEDIA

The growth response in the resazurin method is shown in a column for each concentration of DDS by a G for growth or an I for inhibition. The number of strains is shown in the first column. The source of the strain is shown by the concentration of DDS in the second column and the form of medium (A-agar, B-broth) in the third column. A - indicates not done.

Number of strains	DDS in µg/ml	Medium	DDS in µg/ml					
			0.3	1	2	3	5	10
Parent strain		A/B	I	I	I	I	I	I
2	0.05	A	I	I	-	I	-	I
1	0.075	A	I	I	-	I	-	I
3	0.1	A	-	I	I	I	I	I
1	0.1	A	-	G	I	I	I	I
4	0.1	A	I	I	-	I	-	I
1	0.3	A	I	I	-	I	-	I
1	1.0	A	-	I	I	I	I	I
4	1.0	A	I	I	-	I	-	I
1	2.0	A	-	I	I	I	I	I
1	2.0	B	G	I	-	I	-	I
1	3.0	A	-	I	I	I	I	I
7	10.0	A	I	I	-	I	-	I
3	10.0	A	G	I	-	I	-	I
6	100	B	I	I	-	I	-	I

parent strain -- that is, about 1 in 24,000. Of the four strains, only one was resistant to the concentration of DDS from which it was isolated. The remaining three strains that showed reduced susceptibility to DDS were resistant to 0.3 µg/ml DDS but had been isolated from agar containing 10 µg/ml DDS and had grown from single cell inocula. Although this is not a rigorous measure of the rate of occurrence of DDS resistance in populations of M. kansasii, it does provide the basis for confidence that the results of the experimental program are not the consequence of growth of resistant fractions of the experimental populations.

There remains the question of how susceptible strains arose on media containing DDS. The use of single cell inocula precludes the possibility of interaction of cells within clumps. The possibility that the original colonies did contain resistant cells but that backmutation occurred during subculture cannot be ruled out. However, 5 of 36 isolates retained resistance after subculture. If backmutation is taking place, the rate is very nearly equal to, and perhaps exceeds, the forward mutation rate.

BINDING OF DDS TO CELLS OF M. KANSASII

Ten replicate sets of tubes were prepared to contain DDS in concentrations of 0, 0.1, 1.0, 10, and 100 µg/ml in Dubos broth. The inoculated cultures initially contained 2.5×10^7 cells/ml. The cultures were incubated at 37°C., and the O.D. was measured at 0, 12, 54, and 198 hours.

Aliquots for DDS assay were removed from each culture immediately after the O.D. measurements were made. The maximal volume of sample that could be withdrawn from each tube by pipet was delivered into the chamber of a sterile membrane filter holder fitted with a membrane of 0.15 μ average pore diameter. Gentle suction was applied, and each filtrate was collected separately into a sterile 50 ml Erlenmeyer flask. A 5 ml aliquot was transferred aseptically to a sterile screw cap tube for measurement of the DDS remaining in the medium and stored at 4°C. until assay. Dubos agar slants were inoculated with 0.1 ml portions of the filtrates to demonstrate that no bacterial cells had passed through the filter. These slants were incubated at 37°C. and checked weekly for six weeks.

The filters with the retained organisms were washed in place three times with 5 ml of 1% formaldehyde in 0.85% saline, and were transferred to 25 x 150 mm culture tubes. Ten ml of dichloroethane were added to each tube for extraction, and the tubes were stored at 4°C. until the DDS assays were performed.

The volume of the sample and the O.D. were used to calculate the mass of cells extracted for the DDS determination. Assuming a specific gravity of 1 (see page 37), the volume of cells was also estimated.

In order to test for the possibility that not all of the DDS was being extracted from the cells, parallel sets of filters were prepared at the 198-hour sampling period. One set was treated as above, whereas the second set was subjected to ultrasonic disruption, employing a Sonifier (R) Cell Disruption apparatus at voltage 7 for 15-second periods for a total of 1 minute each. Sonication was

performed in individual 10-ml beakers, with the filters submerged in 3 ml of distilled water and the beakers placed in an ice bath. After sonication, the water was poured into the extraction tubes, and the filter scraps were transferred with forceps to the tubes. The beakers were rinsed five times with 2 ml dichloroethane and the rinses were added to the tubes.

The DDS assays were performed by the method described. The controls included a reagent blank, a drug-free culture filtrate for each day, and a drug-free mass of cells on a filter for each day.

The results of this experiment are presented in Tables 37-39. Because multiplication of the organisms in these cultures was inhibited, the O.D. varied from sample to sample. The volume of culture recovered also varied between 8 and 9 ml. Therefore the data for cell-boung DDS are expressed in μg of DDS per mg of cell mass (Table 37). Unfortunately, binding of DDS to bacterial cells could not be measured at the 12-hour period for the cultures containing 10 and 100 μg/ml DDS because of leakage. None of the cells obtained at 12 hours from cultures containing 0.1 or 1.0 μg/ml DDS bound measurable amounts of the drug.

In the study of recovery of DDS from the culture filtrates (Table 38), there is no indication that M. kansasii destroy DDS. Because the DDS assay method simultaneously measures MADDS, it could also be shown that no DDS is converted to MADDS.

Both the culture filtrate and the cell mass controls produced approximately the same fluorescence as the reagent blanks, so there are no interfering substances produced in the culture.

Table 37

CELLULAR BINDING OF DDS BY M. KANSASII

The amount of DDS assayed in whole cells is shown in µg of DDS per mg of cell mass. The observation period is shown in the first column. The amount of DDS in the cells is shown in a column for each concentration of DDS in the medium from which the sample of cells was taken. The ± 95% confidence limits are shown in parentheses.

Hours of exposure to DDS	DDS in medium in µg/ml			
	0.1	1.0	10.0	100.0
54	0	0	0.081(.01)	0.71(.09)
198	0	0	0.139(.017)	1.47(.18)

Comparison of amount of DDS assayed in whole cells and in broken cells. The observation period is 198 hours. The amount of DDS is shown in a column for each concentration of DDS in the medium from which the sample was taken. The ± 95% confidence limits are shown in parentheses.

Cells	DDS in medium in µg/ml		
	1.0	10.0	100.0
Whole	0	0.139(.017)	1.47(.18)
Broken	0	0.141(.017)	0.477(.056)

Table 38

DDS RECOVERED FROM CULTURE FILTRATES

The amount of DDS assayed in culture filtrates is shown in μg/ml. The observation period is shown in the first column. The amount of DDS is shown in a column for each original concentration of DDS. The ± 95% confidence limits are shown in parentheses.

Hours of exposure to DDS	DDS originally in medium in μg/ml	
	1	10
12	1.08(.09)	10.8(1.3)
54	0.941(.11)	10.12(1.21)

Table 39

ACCUMULATION OF DDS BY CELLS OF M. KANSASII

The ratio of the measured concentration of DDS in cells to the concentration in the medium from which they were taken is shown. The observation period is shown in the first column. The ratio of concentrations is shown in a column for each original concentration of DDS in the medium.

Hours of exposure to DDS	DDS in the medium in µg/ml	
	10	100
54	8.1	7.1
198	13.9	14.7

For the cultures containing 10 µg/ml DDS, the extraction from whole cells and from broken cells (Table 37) gave the same amount of DDS bound. Only one-third the amount was recovered from broken cells as from whole cells recovered from the cultures containing 100 µg/ml DDS. There is no available explanation for this discrepancy.

Table 39 shows the ratio of DDS concentration in cells to that in the medium. DDS is bound against a concentration gradient, and an increasing amount is bound between 54 hours and 198 hours. Measured at 54 hours, DDS is concentrated against a gradient about seven-or-eight-fold by M. kansasii. By 198 hours the ratio of concentration in the cells to that in the medium has increased to 14-to-15-fold. The binding appears to be nearly proportional to the concentration in the media because the ratios at both sampling periods are nearly identical.

The ability of M. kansasii to grow in the presence of DDS can not be attributed to the possession of a permeability barrier that would prevent the accumulation of DDS by the cells. However, no information is available about the subcellular location of the bound DDS.

EFFECT OF DDS ON COLONY FORMATION BY M. SMEGMATIS 607

Quadruplicate corn meal agar plates were prepared containing DDS in the following concentrations: 0, 2, 4, 6, 8, 10, and 12 µg/ml. A broth culture of M. smegmatis 607 was incubated until barely turbid and was then diluted 2 x 10^3-fold in broth. Duplicate aliquots of 0.1 and 0.3 ml of the inoculum suspension were spread on plates of

each drug concentration. Colonies on the incubated plates were counted three times a week.

The results of the experiment are presented in Tables 40-42 and Figure 7. Table 40 shows the total number of colonies on the four plates. Table 41 shows the per cent inhibition represented by the numbers of colonies counted. Figure 7 is a graph of log dose versus response in terms of per cent inhibition. Table 42 shows the MICs for each observation period. The IC50's were determined by interpolation from the log-dose plot.

Inhibition resulted from each concentration of DDS used in this experiment. Inhibition caused by 2 and 4 µg/ml DDS was temporary; 2 µg/ml produced on 1.3% inhibition on day 9, and 4 µg/ml produced only 6.5% inhibition on day 16. At all subsequent observation periods, there was no inhibition from these levels of DDS. At each drug concentration, the per cent inhibition decreased with time. Total inhibition, defined as fewer than 20 colonies, was produced by 4 µg/ml on day 4, by 8 µg/ml until after day 11, and by 10 µg/ml for the duration of the experiment. No colonies appeared on the plates containing 12 µg/ml DDS. The dose-response curves are quite steep. A two-fold DDS concentration difference is associated with a 0 to 50-80% inhibition difference. Finally there is a delay before the appearance of colonies that is dependent upon the concentration of drug. The IC50 was derived by interpolation on the dose-response curve. Using the numbers of colonies visible on day 7, it is 3.14 µg/ml. Derived from the day 32 colony counts, however, it is 5.8 µg/ml. The increase in the value of the IC50 with time

Table 40

EFFECT OF DDS ON COLONY FORMATION BY *M. SMEGMATIS* 607

The number of colonies from four plates are shown for each observation period and each concentration of DDS. The observation period is shown in the first column. The colony counts are shown in a column for each concentration of DDS. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml					
	0	2	4	6	8	10
2	0	0	0	0	0	0
4	818(56)	30(11)	4(4)	0	0	0
7	1037(63)	913(59)	319(35)	35(12)	0	0
9	1285(70)	1268(70)	472(43)	123(22)	0	0
11	1285(70)	1323(71)	643(50)	173(26)	3(3)	0
14	1285(70)	1323(71)	643(50)	202(28)	39(12)	0
16	1332(72)	1351(72)	1246(69)	258(31)	61(15)	2(3)
21	1341(72)	1404(73)	1362(72)	371(38)	92(19)	5(4)
23	1350(72)	1404(73)	1363(72)	460(42)	138(23)	7(5)
28	1350(72)	1404(73)	1363(72)	586(47)	155(24)	7(5)
30	1372(73)	1404(73)	1366(72)	606(48)	163(25)	12(7)
32	1372(73)	1404(73)	1366(72)	611(48)	164(25)	12(7)

Table 41

PER CENT INHIBITION OF COLONY FORMATION BY M. SMEGMATIS

The per cent inhibition is shown for each observation period, and in a column for each concentration of DDS. The observation period is shown in the first column. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml				
	2	4	6	8	10
2	100	100	100	100	100
4	96.3 (94-98)	99.5 (99-100)	100	100	100
7	11.9 (0-23)	69.3 (64-73)	96.6 (95-98)	100	100
9	1.3 (0-12)	63.3 (58-69)	90.4 (89-93)	100	100
11	0 (0-8)	50.0 (43-57)	86.5 (84-89)	99.8 (99-100)	100
14	0 (0-8)	50.0 (43-57)	84.3 (81-87)	97.0 (96-98)	100
16	0 (0-9)	6.5 (0-16)	80.1 (77-84)	95.6 (94-97)	99.8 (99-100)
21	0 (0-6)	0 (0-9)	72.2 (68-76)	93.1 (91-95)	99.6 (99-100)
23	0 (0-7)	0 (0-9)	65.7 (61-71)	89.9 (87-92)	99.5 (99-100)
28	0 (0-7)	0 (0-9)	56.6 (50-62)	88.5 (86-91)	99.5 (98-100)
30	0 (0-8)	0.4 (0-9)	55.8 (50-62)	88.1 (86-91)	99.1 (98-100)
32	0 (0-8)	0.4 (0-9)	55.4 (49-61)	88.0 (86-91)	99.1 (98-100)

Table 42

MIC OF DDS FOR M. SMEGMATIS COLONY FORMATION

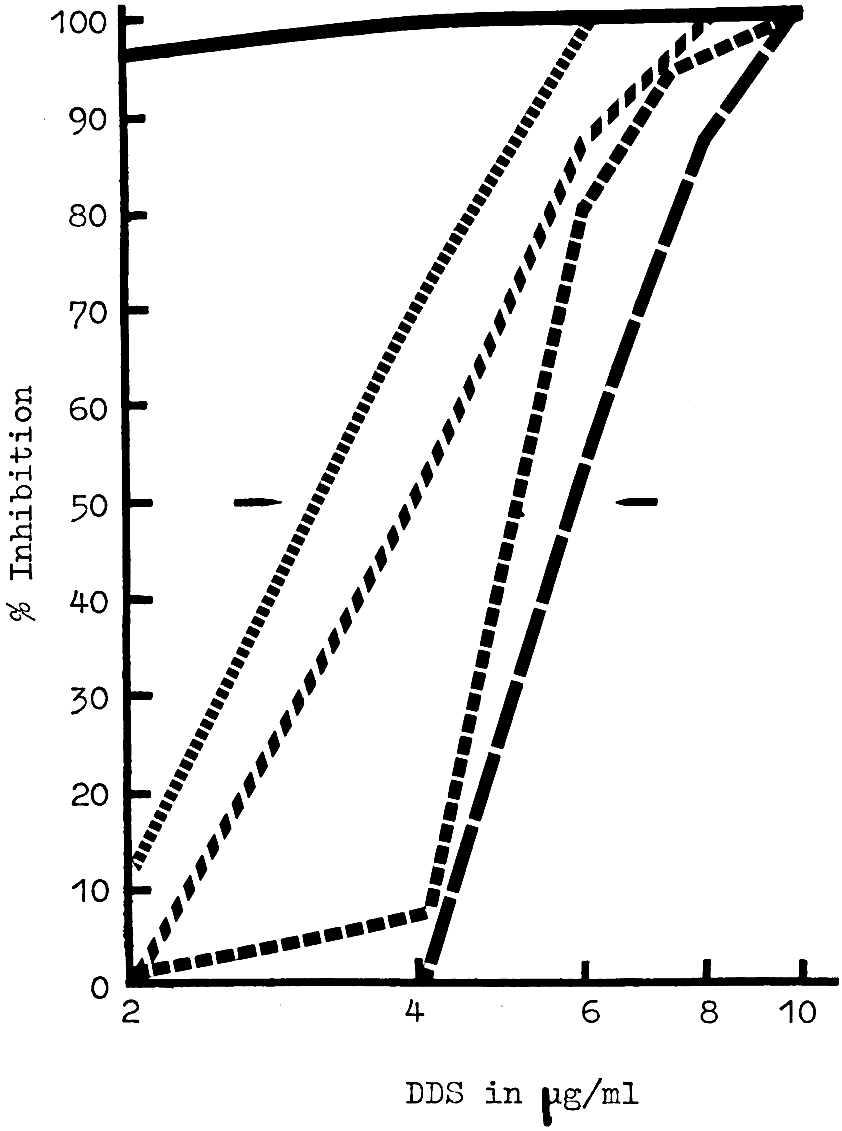
The MIC for each observation period is given in two forms. The first column shows the observation period. The second column shows the IC50 derived by interpolation on the curves in Figure 7. The third column shows the lowest dose that completely inhibited colony formation. The MIC is given in concentration of DDS in µg/ml.

Day	IC50	Total inhibition
4	--	4
7	3.14	8
9	3.45	8
11	4.00	8
14	4.00	10
16	5.06	10
21	5.30	10
23	5.45	10
28	5.70	10
30	5.75	10
32	5.80	10

Figure 7

INHIBITION OF COLONY FORMATION BY *M. SMEGMATIS*

Per cent inhibition is plotted on the ordinate. The $\log_{10}$ concentration of DDS is plotted on the abscissa. The interpolated IC50 is indicated. (▬▬) -- day 4; (┅┅) -- day 7; (⁄⁄⁄⁄) -- day 11; (▪▪▪▪) -- day 16; (▬▪) -- day 32.



appears to approach a limit of 6 μg/ml DDS.

The inoculum of *M. smegmatis* was not composed of single cells for reasons stated in the Methods section. Therefore, the cellular composition of the CFU must have had an influence on these data. The likelihood exists that CFU containing many cells would multiply into visible colonies earlier than single-cell CFU. The effect of variation in CFU composition would be to extend the interval over which the colonies continued to appear.

EFFECT OF INCREASED INOCULUM SIZE ON INHIBITION OF COLONY FORMATION BY *M. SMEGMATIS*

The possibility that increased inoculum size could effectively antagonize the inhibition of DDS was studied in this experiment. Corn meal agar plates were prepared containing DDS in the following concentrations: 0, 10, 12, 14, 17, and 20 μg/ml. A broth culture of *M. smegmatis* 607 was incubated until turbid and diluted 1:2. This suspension was then subjected to three serial 1:10 dilutions. Aliquots of 0.1 and 0.3 ml of each of the dilutions were plated on each of the drug-containing agar plates.

Colonies were counted three times weekly. The number 1000 was arbitrarily selected to be the limit of countable colonies, except for the control plates inoculated with the three most dilute inocula.

For each inoculum concentration and for each drug concentration, Table 43 records the total number of colonies.

In a previous experiment 10 μg/ml DDS was shown to inhibit colony formation by 99.1% (see Table 41). However, the total number

Table 43

EFFECT OF INCREASED INOCULUM SIZE ON INHIBITION OF COLONY FORMATION BY *M. SMEGMATIS*

The number of colonies is shown for each inoculum size and in a column for each concentration of DDS. The inoculum size is shown in the first column, and is shown as the volume of the aliquot in ml times the dilution of the inoculum suspension. The ± 95% confidence limits are shown in parentheses. -- indicates uncountable colonies, i.e., more than 1000.

Inoculum	DDS in µg/ml					
size	0	10	12	14	17	20
0.1 x 1:1000	181(26)	12(7)	4(4)	1(2)	1(2)	62(15)
0.3 x 1:1000	493(43)	0	1(2)	0	2(3)	2(3)
0.1 x 1:100	1872(35)	6(5)	28(10)	11(7)	6(5)	201(28)
0.3 x 1:100	--	2(3)	3(3)	3(3)	0	8(6)
0.1 x 1:10	--	70(16)	434(41)	123(22)	340(36)	--
0.3 x 1:10	--	66(16)	15(8)	6(5)	8(6)	30(11)
0.1 x 1	--	460(42)	--	--	--	--

of colonies counted for that calculation was 12. In this experiment, 30 plates supported countable colonies. The level of inhibition calculated from the 1885 colonies was 99.7%.

The assumption that the small changes in drug concentration did not yield a difference in the number of colonies was tested for significance. The rank-sum test (47) was applied to the medians so as not to require the assumption that the population was normally distributed. This test does require the assumption that the population is randomly distributed, which was tested and accepted at the P = .05 level. The null hypothesis was accepted at the P = .05 level for every concentration of DDS.

The assumption that the large changes in inoculum size did not yield a difference in the number of colonies was tested for significance as above. The null hypothesis was accepted at the P = .05 level for every size of inoculum.

EFFECT OF PABA ON INHIBITION BY DDS OF COLONY FORMATION BY *M. SMEGMATIS*

Three sets of Dubos agar plates were prepared containing both DDS in concentrations of 0, 1.0, and 10.0 μg/ml and PABA in concentrations of 0.1, 1.0, 10.0 100, and 1000 μg/ml. A turbid suspension of *M. smegmatis* was diluted 10^5-, 3 x 10^5-, and 10^6-fold, and the dilutions were employed to inoculate each of the triplicate agars. The colonies were counted on the seventh day of incubation.

The largest number of colonies appeared upon agars containing 1.0 μg/ml PABA. The numbers appearing on the plates containing

0.1 µg/ml PABA were the same whether in the presence of 1.0 µg/ml DDS or no DDS, (χ was 0.56 and 0.17 respectively.) Ten µg/ml DDS was significantly inhibitory in the presence of 0.1 µg/ml PABA. In the presence of 10 µg/ml PABA there was significant inhibition of colony formation, both in the absence of DDS and in the presence of 1.0 µg/ml of the drug. However, there was no significant inhibition of colony formation by 10 µg/ml DDS in the presence of 10 µg/ml PABA (χ = 0.17). When PABA was present in a concentration of 100 µg/ml, colony formation was inhibited by about two-thirds. No colonies appeared in the presence of 1000 µg/ml PABA.

From these results it was concluded that 1 µg/ml PABA in agar did not inhibit multiplication of M. smegmatis 607, and reversed the inhibition produced by 1.0 to 10.0 µg/ml DDS.

EFFECT OF DDS ON M. SMEGMATIS IN STATIONARY CULTURE

Seven sets of tubes were prepared to contain 0, 8, and 40 µg/ml DDS in Dubos broth. The final inoculum concentration was 7,000 CFU/ml. The tubes were incubated at 37°C. O.D. was measured daily.

To examine colony formation, a 0.1 ml aliquot from one tube from each drug concentration was diluted to reduce the concentration of bacteria to a theoretical O.D. = .001. From this dilution, for further serial 1:10 dilutions were made, and each of four agar plates was spread with 0.1 or 0.4 ml volumes of each of these dilutions. The plates contained no drug, PABA 1 µg/ml, or DDS 8 or 40 µg/ml in corn meal agar. Colonies were counted three times a week until the numbers became constant.

Table 44 shows the O.D. measurements for days 0-4, 7 and 9. Inhibition, which was significant beginning on day 2, ranged between 91.7 and 99.7% for both concentrations of DDS.

Figure 8 shows the growth of the control cultures both from O.D. measurements and from CFU measurements, with growth plotted on a $\log_2$ scale. To make the two forms of measurement comparable, the CFU data from day 2 -- those with the narrowest confidence interval -- were chosen. The CFU value was divided by the O.D. measurement for the same sample, yielding a value of about 16,000 CFU/O.D. unit. This factor was used to divide the other CFU values. The curves are essentially identical.

Table 45 shows the number of CFU formed on each plate from each broth culture, except for the results from the plates containing 40 µg/ml DDS. On no occasion did colony formation on agar containing 40 µg/ml DDS differ significantly from zero; the upper confidence limits of the number of colonies on the plates containing the large concentration of DDS approached 0.1% of those on drug-free agar.

In order better to compare the colony formation data, the numbers of colonies were normalized to make the 0 day values each equal to 7.0. This operation is analogous to subtracting the blank from O.D. measurements. These data confirm that there is no net bactericidal effect of DDS under these conditions of inoculum size and drug concentration.

The number of colonies formed by organisms from drug-free broth was inhibited by 1 µg/ml PABA and by 8 µg/ml DDS in agar. The inhibition by PABA and DDS was of the same degree for the day 1 and 2

Table 44

EFFECT OF DDS ON O.D. OF M. SMEGMATIS CULTURES

The O.D. x 10^3 is shown in a column for each concentration of DDS. The observation period is shown in the first column. The ± 95% confidence limits are shown in parentheses.

Day	DDS in µg/ml		
	0	8	40
0	0	0	0
1	2(5)	1(3)	3(3)
2	36(7)	3(4)	3(2)
3	134(34)	4(5)	4(1)
4	273(98)	3(4)	4(2)
7	860(120)	5(6)	3(4)
9	1355(420)	5(11)	4(2)

Figure 8

COMPARISON OF O.D. AND CFU

Growth is shown in logarithmic scale on the ordinate. Time in days is shown on the abscissa. The vertical bars show the 95% confidence range on the O.D. measurements. (▰▰▰▰) -- O.D. x 10^3; (▬▪▬▪) -- (CFU/18) x 10^{-3}.

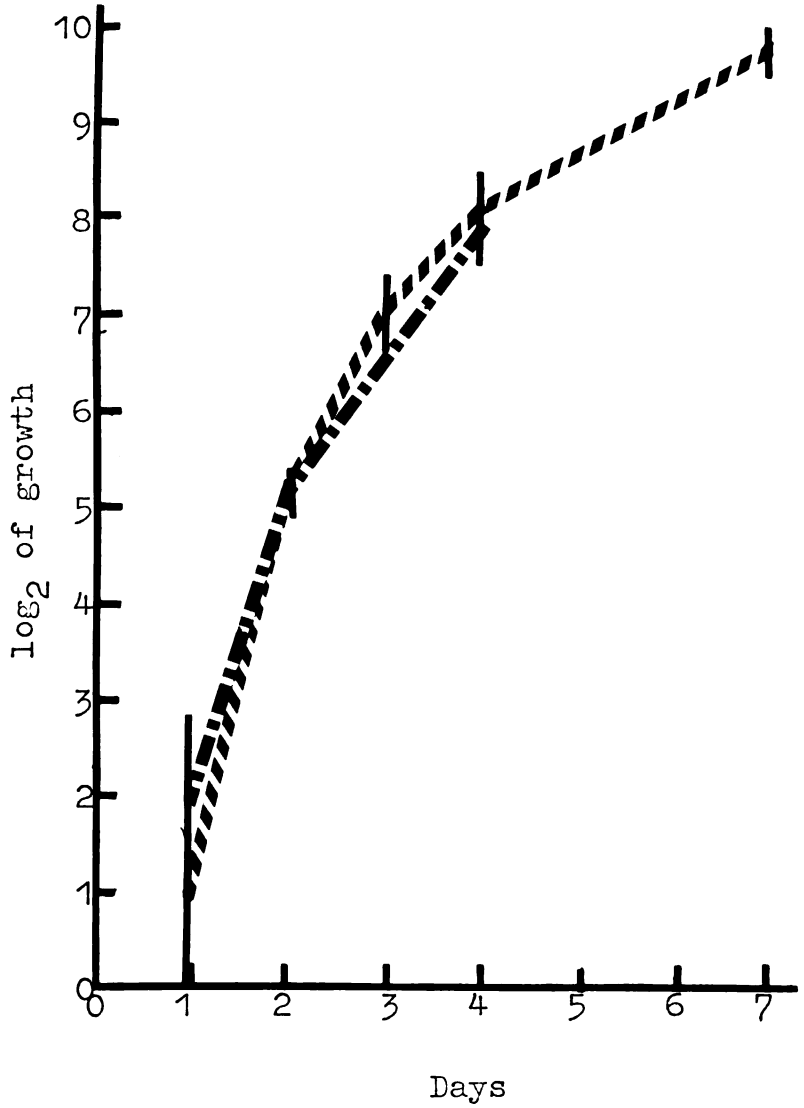


Table 45

COLONY FORMATION AFTER EXPOSURE TO DDS

The number of colonies x 10^{-3} / ml of broth culture is shown for each plate. There is a separate section of the table for the plates from each concentration of DDS in broth. The sampling time is shown in the first column. The colony count is shown in a column for each kind of agar on which the samples were subcultured. The ± 95% confidence limits are shown in parentheses.

Day	Agar containing		
	no drug	1 µg/ml PABA	8 µg/ml DDS
Drug free broth			
0	7.0(3.1)	7.0(2.2)	7.0(2.5)
1	70.8(10.1)	30.6(4.4)	30.8(5.5)
2	649.4(49.6)	132.6(9.4)	135.0(8.0)
4	4589.0(418)	2252.0(192)	787.5(137)
DDS broth 8 µg/ml			
0	7.0(4.4)	7.0(2.1)	7.0(2.6)
1	6.3(9.3)	3.5(1.4)	8.4(2.8)
2	81.8(15)	75.3(6.8)	77.5(8.4)
4	1396.0(287)	1324.0(91)	261.0(15.4)
DDS broth 40 µg/ml			
0	7.0(5.4)	7.0(1.5)	7.0(2.0)
1	21.7(4.3)	5.2(3.7)	5.2(5.4)
2	52.2(14.5)	36.4(3.3)	59.0(6.4)
4	621.0(113)	240.0(19.1)	199.0(4.1)

samples. However, the organisms sampled on day 4 showed greater inhibition by 8 µg/ml DDS than by 1 µg/ml PABA.

The formation of colonies by organisms cultured in broth containing 8 µg/ml DDS showed inhibition at all observation periods and on all formulations of agar. There was no multiplication in the 8 µg/ml DDS-containing broth during the first day of culture. Equal numbers of colonies formed on all three formulations of agar for each observation period with the exception of the day 4 aliquot plated on 8 µg/ml DDS. This sample showed significant inhibition compared to those plated on agar containing no drug or PABA. When the numbers of colonies formed from the broth containing 8 µg/ml DDS are compared to those from corresponding plates inoculated from drug-free broth, there is significant inhibition in every case.

There was also inhibition of colony formation following every period of incubation of cells in broth containing 40 µg/ml DDS. The inhibition was greater than that exhibited by organisms cultured in the presence of 8 µg/ml DDS, except for those cultured on drug-free agar on day 1. Fewer colonies formed on agar containing either PABA or DDS than on drug-free agar, except for the day 2 sample cultured on agar containing 8 µg/ml DDS, for which the number of colonies was the same as that on the drug-free agar.

In order to analyse these relationships more easily, per cent inhibition is presented in Tables 46 and 47. In Table 46, the number of colonies appearing on the drug-free agar is designated as 0 inhibition, and in Table 47, the number of colonies that appeared from drug-free broth is designated as 0 inhibition. The first form of

Table 46

PER CENT INHIBITION OF COLONY FORMATION BY DDS AND PABA

There is a separate section of the table for the plates from each concentration of DDS in broth. The sampling time is shown in the first column. The per cent inhibition with respect to the drug free agar plates is shown in a column for each drug. The ± 95% confidence limits are shown in parentheses.

Day	Agar containing		
	PABA 1 µg/ml	DDS 8 µg/ml	DDS 40 µg/ml
<u>Drug-free broth</u>			
0	0(0-53)	0(0-55)	100(99-100)
1	56.8(42-68)	56.5(40-69)	100(99-100)
2	79.6(76-83)	79.2(76-88)	100(99-100)
4	50.9(41-59)	82.9(78-100)	100(99-100)
<u>DDS broth 8 µg/ml</u>			
0	0(0-57)	0(0-61)	100(99-100)
1	44.4(0-87)	0(0-64)	100(99-100)
2	8.0(0-29)	5.3(0-28)	100(99-100)
4	5.2(0-27)	81.3(75-85)	100(99-100)
<u>DDS broth 40 µg/ml</u>			
0	0(0-56)	0(0-60)	100(99-100)
1	76.0(49-94)	76.0(39-100)	100(99-100)
2	30.3(0-50)	0(0-21)	100(99-100)
4	61.4(49-70)	68.0(60-73)	100(99-100)

Table 47

PER CENT INHIBITION OF COLONY FORMATION AFTER EXPOSURE OF CELLS TO DDS

The per cent inhibition calculated with respect to drug-free broth is shown in a separate section of the table for each concentration of DDS in broth. The sampling time is shown in the first column. The per cent inhibition is shown in a column for each formulation of agar. The ± 95% confidence limits are shown in parentheses.

Day	Agar containing		
	no drug	PABA 1 µg/ml	DDS 8 µg/ml
<u>8 µg/ml DDS in broth</u>			
0	0(0-76)	0(0-47)	0(0-54)
1	91.1(74-100)	88.6(81-94)	72.2(59-85)
2	87.4(84-91)	43.2(33-52)	42.6(32-52)
4	69.6(60-78)	41.2(31-50)	66.9(58-73)
<u>40 µg/ml DDS in broth</u>			
0	0(0-84)	0(0-40)	0(0-47)
1	69.3(57-79)	83.0(66-96)	83.1(58-100)
2	92.0(89-95)	72.5(68-77)	56.3(49-63)
4	86.5(82-90)	89.3(87-91)	74.7(69-79)

calculation should allow the examination of the data in terms of subpopulations within the broth culture that are able to grow in the presence of drug in agar. In this case we find that 1 µg/ml PABA is significantly inhibitory to M. smegmatis following one, two or four days of culture. The cells grown in broth for two days are more inhibited by the PABA than are those which were grown for only half as long or for twice as long. As the inoculum for the broth cultures was a broth culture in logarithmic growth, the meaning and source of these time differences is unknown. There was a subpopulation inhibitable by PABA in the drug-free broth. In the broth containing 8 µg/ml DDS, however, this subpopulation appears to be missing. The inhibition by PABA does not differ from zero. The existence of a subpopulation susceptible to 1 µg/ml PABA in the broth containing 40 µg/ml DDS appears paradoxical. Significant inhibition by PABA occurred among cells cultured in DDS for one day or four days but not among those cultured for two days. The two-day population in drug-free broth was highly susceptible to PABA, but the corresponding population in broth containing 40 µg/ml DDS was susceptible only to a low degree.

A large subpopulation susceptible to 8 µg/ml DDS in agar existed in the drug-free broth. As might be anticipated, this subpopulation was absent from the population of cells in broth containing 8 µg/ml DDS, but only for one and two days of growth. The population present after four days of culture in the broth with DDS contained a subpopulation susceptible to 8 µg/ml DDS in agar that was equal to that in the corresponding drug-free broth. The presence of this subpopulation

in broth containing 40 µg/ml DDS did not follow a uniform pattern. There was a large susceptible population following one and four days in the DDS broth, but none in the population sampled after two days.

The entire population in each broth was susceptible to 40 µg/ml DDS in agar.

The second form of calculation of per cent inhibition allows the examination of the influence of the drug in the broth. Growth occurred in the presence of both 8 and 40 µg/ml DDS. On days 1 and 2, inhibition by 8 µg/ml was about 90%, but dropped to about 70% by day 4. Inhibition in the presence of 40 µg/ml DDS in the broth, however, was only 70% on day 1, but increased to about 90% on days 2 and 4.

In planning this experiment, 1 µg/ml PABA was included in the agar to antagonize the action of any DDS carried onto the agar from the broth cultured cells; i.e., larger numbers of colonies would form on agar containing PABA than on the drug-free medium. The inhibition by PABA of colony formation by cells derived from the drug-free broth was an unexpected finding. However, on days 2 and 4, the per cent inhibition of colony formation from 8 µg/ml DDS onto PABA-containing agar was lower than on day 1; was lower than that on drug-free agar; and was lower than that from 40 µg/ml DDS. There appears to be mutual antagonism between 1 µg/ml PABA and 8 µg/ml DDS. However, 40 µg/ml DDS is not antagonized by 1 µg/ml PABA sufficiently to be demonstrated in this system.

The growth of each of the sub-populations under consideration is illustrated in Figure 9. The growth rates estimated from the curves are shown in Table 48. There was no measurable lag phase in

Figure 9

COLONY FORMATION AFTER EXPOSURE TO DDS

Growth is shown in CFU in logarithmic scale on the ordinate. Time in days is shown on the abscissa. (━━━) -- 0 DDS in broth, 0 DDS in agar; (┃┃┃┃┃) -- 8 µg/ml DDS in broth, 0 DDS in agar; (━ ▪ ━) -- 40 µg/ml DDS in broth, 0 DDS in agar; (▰▰▰▰) -- 0 DDS in broth, 8 µg/ml DDS in agar; (▪━━) -- 8 µg/ml DDS in broth, 8 µg/ml DDS in agar; (■■■■) -- 40 µg/ml in broth, 8 µg/ml DDS in agar.

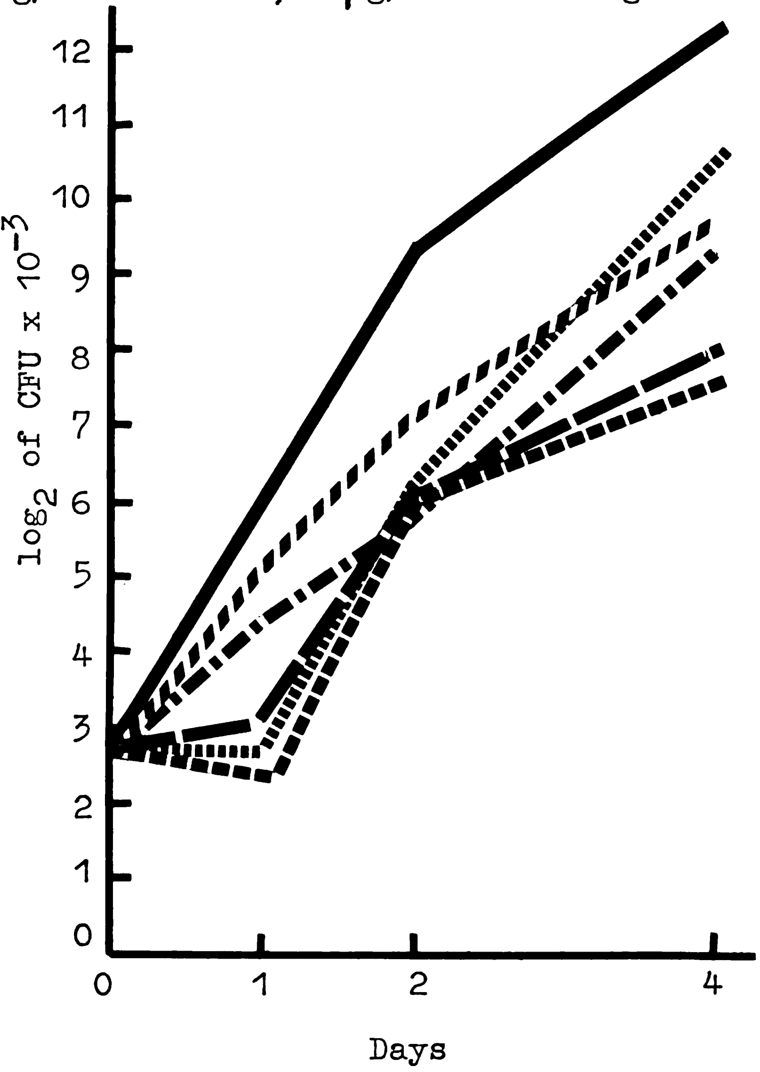


Table 48

GROWTH OF SUBPOPULATIONS EXPOSED TO DDS

The growth rate is shown as the doubling time in hours. There is a separate section of the table for each concentration of DDS in broth. The sampling time is shown in the first column. The doubling time is shown in a column for each formulation of agar.

Days	Agar containing		
	no drug	1 µg/ml PABA	8 µg/ml DDS
<u>Drug-free broth</u>			
0-1	7.2	12.0	12.0
1-2	7.2	12.0	12.0
2-4	16.8	12.0	18.0
<u>DDS broth 8 µg/ml</u>			
0-1	∞	∞	∞
1-2	7.2	5.4	7.8
2-4	12.0	12.0	27.5
<u>DDS broth 40 µg/ml</u>			
0-1	14.4	∞	∞
1-2	14.4	8.4	6.6
2-4	14.4	17.4	28.2

the drug-free broth. The doubling times appear to cluster in four groups: rapid, ranging from 5.4 to 8.4 hours per doubling; intermediate, from 12 to 18 hours; slow, 27 and 28 hours per doubling; and infinite, or no growth. Three of the nine subpopulations multiplied at intermediate rates during the three intervals. Five of the six subpopulations exposed to DDS in broth did not multiply during the first interval, and at a rapid rate during the day 1-to-day 2 interval, followed by a slower rate during the day 2-to-day 4 interval. The day 2-to-day 4 rate was in the intermediate range for the subpopulations growing on agar containing no drug or PABA but slow for those growing on agar containing 8 μg/ml DDS. This is in contrast to the subpopulations from drug-free broth that doubled at the intermediate rate on agars containing drug.

The rate of appearance of colonies was also examined. Among aliquots sub-cultured onto agar containing PABA, there was no significant delay in the appearance of colonies. Neither the concentration of DDS to which the bacilli had been exposed in broth nor the duration of drug contact appeared to affect the rate at which colonies appeared. However, a significant delay was observed among cells exposed to DDS for four days and plated onto DDS agar. The delay lasted for 12 days and was significant at the $P = 0.05$ level.

SUMMARY OF EXPERIMENTAL FINDINGS

DDS had a IC50 for colony formation by M. kansasii of 0.175 or 0.345 μg/ml depending upon the time of measurement. This variation occurs because the primary effect on colony formation is a

concentration-dependent delay in the appearance of the colonies. The delay is particularly striking when single cell inocula are used, as all the colonies appear simultaneously at each drug level. The MIC, measured as total inhibition, similarly shifts with time, no colonies appearing on agar containing 0.5 µg/ml DDS after 60 days of incubation.

The MIC for colony formation by M. smegmatis 607, without the use of single cell inocula, is between 3.14 and 5.8 µg/ml DDS when measured as IC50, again because of the concentration-dependent delay in the appearance of colonies. The MIC, measured as total inhibition, is 8 to 10 µg/ml DDS.

The MIC for M. kansasii in liquid cultures was less than 0.05 µg/ml. The pattern of inhibition for cultures grown in tubes shaken three times a week shows a delay of a few days before the onset of inhibition followed by an increasing degree of inhibition that is a concentration-dependent decrease in the growth rate. For liquid cultures grown in constantly shaken flasks, there appears to be immediate onset of inhibition in the form of a prolongation of the lag phase, followed by the establishment of the pattern found in the stationary cultures. The bacteriostatic effect of reduction in growth rate is consistent with the delay in colony formation.

A concentration-dependent delay in colony formation has been shown for cells cultured in broth containing DDS and then subcultured onto drug-free agar, for both M. kansasii and M. smegmatis. The delay in colony formation is also dependent upon the duration of the exposure to DDS.

Bactericidal activity has been shown in liquid cultures containing small inocula, but only for concentrations of DDS representing at least 100-fold the MIC.

Although the occurrence of resistant mutants was demonstrated, it is not a significant factor in the interpretation of these experimental findings.

DDS is accumulated against a gradient by cells of M. kansasii in liquid culture. The concentration increases with time.

PABA is inhibitory to both M. kansasii and M. smegmatis at a concentration of 10 µg/ml. Total reversal of DDS inhibition by PABA was demonstrated for M. kansasii with 0.8 µg/ml DDS and 1.0 µg/ml PABA, and for 0.1 µg/ml DDS and 0.1 µg/ml PABA. Total reversal for M. smegmatis occurred with 10 µg/ml DDS and 1 or 10 µg/ml PABA. Total reversal for M. kansasii was also demonstrated by the resazurin method for 10 µg/ml DDS and 1 µg/ml PABA. The concentrations of PABA that reverse the inhibition of DDS border on the inhibitory range of PABA itself for these two organisms. The concentration-and duration-dependent delays in colony formation observed with cells exposed to DDS in liquid culture were not observed when M. smegmatis was plated onto agar containing PABA.

CHAPTER VI

DISCUSSION

Experiments which measured the effect of DDS on colony formation were performed with both M. kansasii and M. smegmatis. The IC50 for M. kansasii was 0.175 µg/ml using a one month end-point and 0.345 µg/ml at two months. The IC50 for M. smegmatis was 4.0 µg/ml for a two week end-point, and 5.7 µg/ml at four weeks.

The MIC of DDS for M. kansasii has been reported only by Pattyn and van Ermengem (74), from whom the strain used in these studies was obtained. They used a drop-plate method on LJ medium, and scored the growth using the assumption that confluent growth equalled 200 colonies (72). The incubation period was not stated. These authors reported that this strain was inhibited by 0.3 µg DDS/ml, but not by 0.1 µg/ml. The identity of the MICs is interesting, because the methods and media were so different.

Karlson (51) reported that 12.5 µg/ml, but not 3.1 µg/ml DDS inhibited one strain of M. smegmatis. This result, arrived at by colony counts on egg-yolk agar after two weeks of incubation, compares well with the present finding that 10 µg/ml prevented the appearance of colonies by day 14. Two strains of M. smegmatis were tested by Pattyn and van Ermengem. One strain was not susceptible to 3 µg/ml DDS. An equivalent result was obtained in the present study at seven days for 2 µg/ml and at sixteen days for 4 µg/ml. The other strain was reported to be uninhibited by 0.3 µg/ml but "2% resistant" to 1 and 3 µg/ml DDS. If this means 98% inhibition, then an IC50 of 0.56 can be estimated for this strain. Naylor and Hanks (69) may or

may not have been using a strain of M. smegmatis. They identified it as M. ranae obtained from Trudeau. Gordon and Smith (39) identified the strain of M. ranae that they received from Trudeau as belonging to the species M. smegmatis. However, other strains of M. ranae have been identified as M. fortuitum (38,107). The strain of M. ranae studied by Naylor and Hanks was inhibited by 1 to 4 µg/ml DDS at five days, but by 0.3 µg/ml when observed after only two days of incubation.

With both M. smegmatis and M. kansasii, the dose-response curves were very steep. The concentrations of DDS were arranged in a three-fold series for M. kansasii, while a two-fold series was used in the study of M. smegmatis. With M. kansasii, 0.1 µg/ml DDS produced 0% inhibition at the same time that 0.3 µg/ml produced 100% inhibition. Subsequently, 0.3 µg/ml produced 33% inhibition while 1.0 µg/ml (and even 0.5 µg/ml) produced 100% inhibition. During all the observation periods in which M. smegmatis was not inhibited by 2 µg/ml DDS, 6 µg/ml produced 55 to 90% inhibition. Similarly, during the observation periods in which 4 µg/ml produced essentially no inhibition, 12 µg/ml produced 100% inhibition. This confirms Karlson's (51) observation that there is "a sharp end-point of growth" with DDS using four-fold concentration differences, and contradicts Naylor and Hanks (69) who observed "the inability of this drug to produce clear-cut growth inhibitory end-points."

A concentration-dependent delay in the appearance of colonies was observed with both M. kansasii and M. smegmatis. The only difference between the results with the two species was that the

colonies of M. kansasii appeared simultaneously, whereas those of M. smegmatis appeared more gradually. This difference in results may be attributed to differences in experimental design rather than to interspecific variation. The inoculum of M. kansasii was composed of single cells whereas that of M. smegmatis was a suspension of cells, many of which were in clumps. Theoretically, the larger the number of bacilli that contribute to the formation of a single colony, the more rapidly that colony would appear. The report of Naylor and Hanks (69) of larger inhibitory concentrations following longer periods of incubation may be interpreted as a concentration-dependent delay in colony formation. They used a massive inoculum. Cells in clumps may interact to counteract the effect of DDS; the interaction would be evident as a concentration-dependent delay in the appearance of colonies, so that colonies arising from larger clumps would be expected to appear earlier than those from smaller clumps.

One wonders whether bacilli can be plated in sufficiently large numbers so that they are in sufficient proximity to interact as do cells in clumps. There is reason to suspect that such might be the case. Naylor and Hanks (69) reported that "slight growth occurred even at drug concentrations of 60 μg/cc," using an inoculum of 10^8 cells of M. ranae or M. phlei which was somewhat inhibited by 0.3 μg/ml DDS. Collins et al (15) reported that as much as ten times the concentration of PAS* was needed to inhibit M. tuberculosis on Steenken-Smith medium as on LJ medium, but the trend was reversed

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*Similarities of DDS and PAS are discussed subsequently.

if the inoculum used in the drop-plate method was reduced from 10^6 to 10^3 AFB. Also, this author had attempted to use the replica plate method of Lederberg (56) to detect resistant mutants among colonies that appeared on agar containing DDS. The colonies on the control agar had appeared at 14 days, and at 20 days on the agar containing 0.1 µg/ml DDS. The replica plates produced large colonies in four days, and were equivalent in the presence and absence of DDS. They were not drug-resistant, nor were they contaminants. It appeared that the large number of cells inoculated onto the area for each colony was obscuring the previously-observed inhibition.

Experiments with both *M. kansasii* and *M. smegmatis* were done to determine if large inocula would allow bacilli to interact in antagonising inhibition of colony formation by DDS. In both cases, the inocula were large enough to produce confluent growth in the absence of inhibition. The number of bacilli inoculated would have produced an average surface density of 76 bacilli/mm^2 of *M. kansasii* and 147/mm^2 of *M. smegmatis*. The DDS concentrations were slightly larger than the concentration that produced total inhibition in the previous experiments. *M. kansasii* produced no colonies at all. Colonies of *M. smegmatis* appeared, but represented 99.7% inhibition. In these experiments, again, the inoculum of *M. kansasii* was composed of single cells, whereas that of *M. smegmatis* was a suspension containing clumps. In this experiment, cells in clumps appear to interact to antagonize the action of DDS. However, up to the point at which colonies representing 0.3% of the inoculum become too numerous to count, increasing the size of the inoculum does not place the bacilli

in sufficiently close proximity to antagonize the action of DDS. This interpretation would explain the failure of Naylor and Hanks (69) to observe "sharp end-points."

Inhibition of the growth of *M. kansasii* in liquid culture was produced by concentrations of DDS lower than required to inhibit colony formation. The IC50 DDS for *M. kansasii* in liquid culture was not determined because the lowest concentration tested, 0.05 µg/ml, produced more than 50% inhibition. Lower concentrations were not tested because the growth in the presence of 0.05 µg/ml showed much greater variation than the growth in the presence of any other concentration of drug, and because there appeared to be a trend of greater variation with lower drug concentration (Table 23). The highest concentration used in the same experiment, 0.8 µg/ml, did not produce total suppression of growth. Even 10 µg/ml DDS in shaken culture did not produce complete bacteriostasis. One may wonder which is more applicable to the *in vivo* situation? The inhibitory concentration indicated by the great inhibition produced by 0.05 µg/ml DDS puts the susceptibility of *M. kansasii* much closer to that measured for *M. leprae* in the mouse--0.02 µg/ml (93). The observation of different MIC's on liquid and solid media is not limited to *M. kansasii*. The MIC for colony formation by *M. smegmatis* varied from 3 to 5.8 µg/ml DDS according to the length of incubation. Using the resazurin method, 1 µg/ml in broth was inhibitory while 0.3 µg/ml was not. Morrison (64) reported an MIC of 0.5 µg/ml in broth for the parent strain of the culture of *M. smegmatis* used in the present study. (It has been transferred no more than three times in this laboratory.)

This observation that much less DDS is needed for 50% inhibition in broth than in agar suggests an explanation for an observation of Karlson (50), who reported that DDS administered to mice at levels known to produce a concentration of 10 to 30 µg/ml in whole blood (100, 104) was effective against disease caused by *M. fortuitum* and *M. microti*. These strains, however, were not susceptible to inhibition of colony formation by 100 µg/ml DDS.

The results of studies of DDS inhibition of growth in shaken versus stationary liquid cultures of *M. kansasii* indicated that there were differences in the initial stages of the cultures. In stationary cultures that were agitated briefly on a variable-speed mixer three times weekly immediately prior to the measurement of O.D., growth started immediately in all tubes, with inhibition becoming measurable only after a week. Conversely, in shaken cultures, DDS appeared to induce a lengthening of the lag phase. Following these initial differences, the effect of DDS was the same---a concentration-dependent decrease of the growth rate.

Naylor and Hanks (69) observed a "lag in the production of sulfone effect" with *M. phlei* and *M. ranae*. Shepard (96) also observed that an interval elapsed before DDS was able to stop growth of *M. leprae* in the mouse. The demonstration of this effect in a highly sensitive strain of *Mycobacterium in vitro* confirms that the delay is specifically a drug-parasite interaction and is not brought about by the host.

Although no one has reported the effect of shaken versus stationary culture on the dynamics of drug-inhibited mycobacterial

populations, many have reported differences in growth of uninhibited cultures. *M. tuberculosis* (54,62,90) and *M. avium* (62) produced much less growth in shaken cultures than in those that were stationary, whereas *M. ranae* was stimulated in shaken culture (62). Volk and Myrvik (112) found that logarithmic growth occurred in shaken cultures of BCG, *M. phlei*, and *M. smegmatis*, whereas arithmetic linear growth was observed in the stationary cultures. Halpern and Kirchheimer (41) determined that either form of growth was reestablished merely by the initiation or cessation of aeration by direct bubbling of oxygen. Fisher and Kirchheimer (31) observed that the typical form of growth for 15 strains of *Mycobacteria*, representing ten species, was linear arithmetic growth. *M. kansasii* was not among the strains tested by them. In the present report, the growth of *M. kansasii* in both stationary and shaken cultures was logarithmic (Figures 3 and 4). The growth rate, of course, was much faster in the shaken cultures. This is consistent with the finding that with intervals up to the highest studied--32 hours, the growth rate correlated exactly with the frequency of shaking. No difference in correlation was found in the presence of DDS or in its absence.

Youatt and Tham (124) mention that inhibition of BCG by INH only occurs under aerobic conditions. Naylor and Hanks (69), using the Warburg method, had determined that DDS did not inhibit *Mycobacteria* by interference with respiration.

The binding study showed that the amount of drug taken up by *M. kansasii* is dependent on both the concentration of DDS and the duration of exposure. The rate of appearance of colonies on drug-free

agar following exposure of the bacilli to DDS in broth was also dependent both on the concentration of drug and on the duration of contact. Therefore, it seems that the effect of the increased duration of contact is merely that the organism accumulates a greater amount of drug. A finite period of time necessary for the organism to bind an inhibitory amount of drug may be observed as a delay in the expression of inhibition in stationary cultures.

Others have observed a delay in the appearance of colonies, or a lag before the resumption of multiplication following the transfer of Mycobacteria from drug-containing to drug-free medium. Shepard (92) reported that DDS inhibited M. leprae in the mouse, and that the growth was delayed beyond the termination of drug administration by 26 to 33 days. This delay was in addition to any delay that could have resulted from the slow excretion of the drug by the mice. The length of the delay was attributed to the time necessary for the surviving population, multiplying at a normal rate, to replace those killed by the drug. Those killed were estimated to be 77 to 84% of the population.

Barclay and Winberg (3) observed that M. tuberculosis exposed to INH also exhibited a lag before the multiplication resumed as measured by O.D. They, too, proposed that the delay represented the time needed for the surviving bacilli to regrow to the original population density.

Levy (58) observed a "marked lengthening of the time to plateau during the first week of DDS..." administration to mice infected with M. leprae in the foot-pad. This was found for cells

sub-inoculated from the drug-treated animals to animals not exposed to the drug, and is therefore most analogous to a delay in the rate of colony formation for bacilli subcultured from drug-containing broth onto drug-free agar. The data supported 2 alternate explanations: 1) that there is rapid killing of a large susceptible population, leaving a resistant population; or 2) the population was homogeneous with respect to DDS susceptibility, and the DDS treatment uniformly lengthened the lag phase of multiplication. The first explanation follows the reasoning of Shepard and of Barclay and Winberg. However, it added the idea that the survivors must be resistant. This would indicate that 10% of the population was resistant to a DDS concentration 1000-fold the MIC. Therefore, the second explanation seemed more reasonable. Unfortunately, a direct measurement of the lag phase is not possible in the mouse foot-pad method.

Dickinson and Mitchison (16) studied the inhibition of M. tuberculosis with each of six drugs in broth. Following one and four days of exposure of the cultures to drug, the medium was replaced by drug-free broth. Samples were regularly plated for determination of viable units. For thiacetazone and thiocarlide, the number of viable units began to increase immediately following the termination of drug exposure. However, the multiplication following exposure to thiacetazone was conspicuously slower than that of the controls. With INH, ethionamide, cycloserine, and streptomycin on the other hand, the number of viable units either remained constant (INH-one day), or decreased and then remained constant for a significant

period of time following the termination of drug exposure. The populations that regrew were not resistant to the drug. If the survivors had been resistant, back mutation would have had to be complete in six to nine generations. It appears likely that the colony counts were done following a standard incubation period as that is the standard method.

Duerr (25) observed the time of appearance of growth of the initial isolates of M. tuberculosis from patients under treatment with PAS, INH, and streptomycin. The cultures became positive at a variety of times throughout the observation period of 30 weeks. Parallel cultures, on medium containing 10 µg/ml PABA, developed colonies in a higher percentage of samples by the four-week observation period than those on identical medium without PABA did in 30 weeks. This indicates that there were "viable units" in some of the samples that were unable to form colonies on routine media during routine incubation periods.

In summary, bacilli that multiply following exposure to inhibitory drugs need not be resistant to the drug. They may multiply more slowly than those not exposed, and may exhibit a prolonged period of nonmultiplication followed by multiplication. Which of these effects obtains with a given drug depends on the nature of the drug. Any of these effects could have contributed to the observations on DDS inhibition of M. leprae in the mouse.

DDS slows the growth rate of cultivable Mycobacteria. Because slowing of growth rate could produce as much lengthening of the time to plateau as would a prolonged lag phase, there is no reason to

exclude such an explanation in the studies of M. leprae. However, direct documentation of a slowing of growth rate must occur in a host less limiting to normal multiplication of M.leprae. Perhaps it can be done in the immunosuppressed mouse, or in the armadillo when a strain consistently susceptible to progressive infection is developed.

Dickinson and Mitchison (16) concluded that the period of nonmultiplication represented the time necessary for surviving cells to recover from the damage induced by the drug. Duerr (25) proposed a more specific hypothesis. She proposed that bacilli that had been exposed to drug brought bound drug to the otherwise drug-free medium. The bound drug continued to be inhibitory to the cell in which it was located until one of the following four circumstances intervened: 1) death of the organism; 2) chemical alteration of the inhibitor to a non-toxic molecule; 3) diffusion of the inhibitor out of the organism; or 4) diffusion of the natural antagonist into the organism. The first circumstance would result in no colony, but the remaining three would result in colonies which would appear after a delay.

M. kansasii accumulated DDS and therefore Duerr's hypothesis can be applied. M. kansasii could not be demonstrated to alter DDS in any way, thus eliminating detoxification as a significant mechanism for the resumption of its multiplication. This organism does not depend on an exogenous source of PABA, which therefore is not specifically provided in the medium. The amount in Dubos medium is probably less than 0.002 µg/ml, and the contribution of antagonist to the termination of the nonmultiplying state would therefore depend on endogenous synthesis. However, any growth of bacilli in the

presence of DDS resulting from significantly increased synthesis of PABA would be recognized as resistance. This mechanism of resistance has been reported only for *Staphylococcus aureus* strains resistant to sulfonamides (55). Failure to find resistance to sulfonamides by such a mechanism has been reported for *Escherichia coli* (71) and *D. pneumoniae* (120). It is therefore doubtful that antagonist plays a significant role in the termination of the nonmultiplying state.

The reduction of the amount of bound drug by diffusion remains to be considered as the explanation for the resumption of multiplication of *M. kansasii* following exposure to DDS. An additional mechanism for reducing the concentration of drug in bacilli must be considered for DDS, as inhibited populations do undergo cell division. One division cycle dilutes the amount of drug per bacillus by half. As growth of *M. kansasii* was not completely suppressed by 10 µg/ml DDS, it is possible that eight divisions could take place, which would still leave a significantly inhibitory concentration, if this were the sole means of reducing drug concentration.

Methods of observing the dynamics of bacterial populations following termination of exposure to inhibitors differ with respect to the information they provide. Neither the measurement of O.D. nor the mouse foot-pad method can distinguish regrowth from a small surviving population from the resumption of multiplication by a larger non-multiplying surviving population. Serial viable counts, if a single end-point count is used, will tend to underestimate the number of viable units. Repeated counts of colony formation provide the most detailed information concerning the number of surviving

bacilli, and the time course of the resumption of multiplication. Underestimation of the number of surviving bacteria will result in an overestimation of the degree or existence of a bactericidal effect, an overestimation of the rate of growth following the nonmultiplying state, and an overestimation of the contribution of resistance, should any resistant mutants be found in the resulting population.

PABA proved to be inhibitory for both M. kansasii and M. smegmatis. In broth cultures the pattern of inhibition was identical to that of DDS. At approximately equal concentrations, PABA antagonizes the inhibition of DDS, up to a limit represented by the inhibitory concentration of PABA.

Hedgecock (44) reported the effect of 2 mycobacterial metabolites of PABA (106) on M. kansasii. The first compound formed, para-aminobenzyl alcohol, inhibited one of three strains of M. kansasii at a concentration of 10 µg/ml, whereas the second, para-hydroxy-aniline, inhibited two of three strains at the same concentration. Furthermore, PAS inhibition was reversed by increasing concentrations of the PABA metabolite, except that for two of three concentrations of PAS in each of two strains of M. kansasii, there was less growth in the presence of 10 than of 5 µg/ml of the PABA metabolite.

Shepard (93) reported that PABA partially antagonized the inhibition of M. leprae by the minimal effective mouse dose of DDS. Calculation of per cent inhibition from his data indicate that the average number of AFB in the DDS group was 11% of the number in the group receiving both DDS and PABA. However, the average number of

AFB in the group receiving PABA alone was 22% of the average number of AFB among three groups receiving no drug at all. Therefore, the possibility exists that PABA is inhibitory to M. leprae as well as M. kansasii. The concentration of PABA in the blood of the mice was found to be 3-4 µg/ml.

The reversal of DDS-inhibition by PABA has been reported for M. ulcerans, M. gastri (74), M. tuberculosis (43), BCG (20,128), M. smegmatis 607 (68), and M. phlei (69).

Of the drugs in regular use against Mycobacteria, PAS probably bears the greatest similarity to DDS. The spectra of susceptibility of M. kansasii to DDS and PAS are similar. Karlson (51) reported that nine of 20 strains of M. kansasii were susceptible to 3.1 µg/ml DDS, and seven were susceptible to 12.5 µg/ml. Pattyn and van Ermengem (74) reported that eight of 17 strains were susceptible to 0.3 µg/ml and five to 1.0 µg/ml, whereas four were not susceptible to 3 µg/ml. Wichelhausen and Robinson (119) reported that three of 281 strains were susceptible to 5 µg/ml PAS, and 129 were susceptible to 25 µg/ml. Wolinsky et al (121) reported that eight of nine strains were susceptible to 10 µg/ml. Jones and co-workers (49) reported six of 30 strains to be susceptible to 2 µg/ml. Hedgecock (44) reported MIC's of 3, 4, 5, and 0.5 µg/ml PAS for four strains of M. kansasii.

PABA reverses the inhibition of M. kansasii produced by either DDS or PAS. In the present report, 0.6 µg/ml PABA produced a 50% reversal of the inhibition produced by 0.8 µg/ml DDS. Pattyn and van Ermengem (74) reported that 0.9 µg/ml PABA in the presence of 3 µg/ml

DDS allowed the appearance of 50% of the number of colonies in the control culture for a strain of M. kansasii with an MIC of 0.3 µg/ml.

Hedgecock (44) reported the inhibition indices of PAS/PABA for each of four strains of M. kansasii. At the MIC's of 3, 5, 4, and 0.5 µg/ml PAS that he reported, the reversing concentrations of PABA may be calculated to be 0.6, 2.5, 0.4, and 0.25 µg/ml PABA respectively. However, he also reported O.D. data for inhibition in an enriched Kirchner's medium that was quite stimulatory to the uninhibited organisms when compared to the Kirchner's medium in which the previous values were obtained. One may recalculate his data, using the methods of the present report. The lowest concentration of PAS used, 5 µg/ml, produced 70 to 80% inhibition of three strains of M. kansasii. Whereas the fourth strain was inhibited 33% by this concentration of PAS, 100 µg/ml only produced 37% inhibition. No O.D. measurements were included in this paper for the reversal of PAS inhibition by PABA, but the mycobacterial metabolites, p-aminobenzyl alcohol and p-hydroxyaniline (106), showed similar antagonist properties. Ten µg/ml p-aminobenzyl alcohol reversed the inhibition of 9 and 12 µg/ml PAS by 50% for two strains. From another set of Hedgecock's data, it may be seen that inhibition produced by 5 µg/ml PAS was 50% reversed by 1.8 µg/ml p-aminobensyl alcohol; inhibition produced by 10 µg/ml is reversed by 3.6 µg/ml p-aminobensyl alcohol; and inhibition produced by 20 µg/ml PAS was reversed 50% by 4.4 µg/ml of the PABA metabolite.

Karlson (51) stated that there was no cross-resistance between PAS and DDS for M. tuberculosis. However, this statement seems to

be based on the observation that nine strains that already exhibited multiple resistance to INH, PAS, and streptomycin, were inhibited by some concentration of DDS. The fact that parent and mutant strains were not compared, and that none of the PAS-resistant strains was susceptible to the lowest tested concentration of DDS, leaves this question open.

A final similarity of DDS and PAS is that both drugs are ineffective when used to treat mice experimentally infected with M. kansasii (45,50), although these strains are susceptible in vitro to concentrations of the drugs lower than those attained in the blood of the mouse. Hedgecock (44) studied the reversal of PAS inhibition of his strains in vitro, and concluded that PAS inhibition was reversed by methionine and oleate available in the serum. Although there was significantly more growth in the presence of methionine, oleate, and/or serum in cultures inhibited by PAS, there was also significant stimulation by these materials in uninhibited cultures. No one or combination of these materials completely abolished the inhibition by PAS. PABA or its mycobacterial metabolites were the only compounds tested which could do that.

The ability of M. kansasii to utilize PABA from its surroundings cannot be the explanation. Pattyn and van Ermengen (74) reported a DDS/PABA inhibition index for M. kansasii of 1-3. Yet for M. ulcerans, which has a DDS susceptibility almost identical to M. kansasii and is effectively inhibited in the mouse by DDS (29), the inhibition index was 500-2000. Vastly smaller amounts of PABA are needed to antagonize the inhibition of M. ulcerans than of M. kansasii.

A difference between DDS and PAS is the lack of effect of the latter compound on infections by *M. leprae* (92) or by *M. ulcerans* (29) in the mouse.

Thus we have two drugs that, by structural similarity, should be able to interact with the same enzymatic and/or binding sites as a natural biosynthetic product and substrate which itself has very limited functions, and can itself become inhibitory at high concentrations; yet, they can be distinguished by some strains of *Mycobacterium*. Either the nature of the biosynthetic machinery in use, or the nature of milieu, allows inherent susceptibility to be masked, or inherent resistance to be circumvented for some species, when their susceptibilities are compared *in vitro* and *in vivo*. This should provide an elegant model system for comparative studies of host influences on permease systems, or for high resolution of binding sites on permeases or biosynthetic enzymes.

Although PAS bears more resemblance to DDS than does INH, some comparisons with the latter will be made because INH has been studies in terms of intermittent regimens *in vitro*, whereas PAS has not.

Youatt and Tham (125) and Beggs and Jenne (4) studied the uptake of INH using the C^{14}-labeled compound. They found that the tubercle bacillus can concentrate INH against a gradient. The degree of accumulation is dependent upon the concentration of the drug in the medium. The ratio between the external and internal concentrations becomes less as the concentration in the medium is increased. Youatt and Tham found the uptake from medium containing 10 μg/ml to be

138 µg/g bacterial mass, whereas that taken up from medium containing 100 µg/ml was 370 µg/g. This maximum was reached by four to six hours.

For comparison, after four days M. kansasii had accumulated 81 µg/g DDS from medium containing 10 µg/ml, and 710 µg/g from medium containing 100 µg/ml. The uptake had not yet reached a maximum. At eight days, the accumulation from the 10 µg/ml medium had reached 139 µg/g and that from 100 µg/ml had reached 1470 µg/g.

Beggs and Jenne reported uptake only in terms of the ratios of external to internal drug concentration. For comparison, Youatt and Tham reported ratios as follows: for 100 µg/ml INH the ratio was 1:1.02, and for 10 µg/ml the ratio was 1:4.6. Beggs and Jenne measured uptake at lower concentrations: 1.0, 0.1, and 0.01 µg/ml INH. The maximal uptake was not achieved by 7.5 hours, but was by 24 hours at these concentrations. The ratios of external to internal concentration at 7.5 hours and 24 hours for medium containing 1.0 µg/ml INH were 1:17 and 1:21; for 0.1 µg/ml they were 1:26 and 1:30; and for 0.01 µg/ml, they were 1:31 and 1:46 respectively.

The ratios of external to internal concentration for DDS were intermediate between those of the high concentration studies of Youatt and Tham and the low concentration studies of Beggs and Jenne. For medium containing 100 µg/ml DDS the ratio was 1:7.1 at 54 hours and 1:14.7 at eight days. The ratios for medium containing 10 µg/ml DDS were 1:8.1 and 1:13.9 respectively. For medium containing 1 µg/ml, the amount accumulated by the cells was too small to permit measurement. If one assumes that the ratios in the presence of

1 μg/ml would be no more than two- or three-fold different from those observed in the presence of 10 μg/ml, then the theoretical values are also too small to be measured. The drug concentrations reviewed here are in similar relation to the MIC's for both drug/parasite systems.

The ability of a pathogenic bacterium to concentrate drug from the surrounding medium suggests one mechanism by which intermittent chemotherapy works. INH has been shown to be an effective agent for intermittent chemotherapy of _M. tuberculosis_, in mice (5,46), in guinea pigs (17) and clinically (34,110). Dickinson and Mitchison (16) state "the suitability of a drug for intermittent chemotherapy depends on its ability to inhibit bacterial multiplication between doses when a bacteriostatic concentration is no longer present, and to a lesser extent, on its bactericidal activity." The binding data suggest that the bacilli contain a large concentration of drug at the time drug exposure is terminated.

Based solely on the observation that DDS is taken up more slowly than INH, it is probable that two or three days of drug administration alternating with a few days of no drug would be a more effective intermittent schedule with DDS, than an intermittent schedule of single doses. The additional duration of contact would provide additional time for the bacilli to bind higher concentrations of DDS.

As opposed to DDS, INH is chemically altered by _M. tuberculosis_. Youatt and Tham (125) reported that much of the C^{14} radioactivity that they recovered from bacterial cells incubated with C^{14}-INH was no longer INH. Isonicotinic acid accounted for approximately 50-60%

of it, with 4-pyridyl methanol accounting for another 20%. These compounds were not inhibitory. Therefore, of the three mechanisms for termination of the non-multiplying state that were suggested by Duerr (25) and discussed above, one--detoxification--available to bacilli inhibited by INH that is not available to bacilli inhibited by DDS. This may indicate that inhibition continuing after removal of drug is more gradually terminated when induced by DDS than when it is induced by INH.

Binding and inhibition are not inseparable. Youatt and Tham (124) reported that although little INH is bound by INH-resistant Mycobacteria, the drug freely enters into both resistant and sensitive cells. In the susceptible cells, the drugs binds to an enzyme that is lacking in the resistant cells. Although there is inhibition when there is binding, Peiser and co-workers (76) reported inhibition by 0.5 to 25 μg/ml INH of M. tuberculosis strains resistant to 100 μg/ml INH. The inhibition that they observed was a prolongation of the lag phase of growth in liquid culture.

Similar inhibition by the drug to which strains of M. tuberculosis have demonstrated resistance was reported by Zykov and co-workers (129). They reported that growth appeared more slowly on L-J medium containing PAS or thiacetazone than on drug-free medium when strains resistant to the respective drug were tested.

So we have a situation in which several drugs including DDS may produce clinically useful inhibition by slowing the growth of the parasite, or by prolonging its lag phase; yet these drugs will not prevent the appearance of colonies on test medium containing that

drug. Because clinical laboratory methods are designed to demonstrate the prevention of the appearance of colonies, clinically useful inhibition may be overlooked. It is suggested that inhibition of growth in broth, most simply done with the resazurin method, be tested routinely in addition to susceptibility tests on solid medium. This is especially recommended for the mycobacterial human pathogens of Runyon's Group III (87) for which treatment failure with standard anti-tuberculosis drugs occurs as often as not, and for which there are no other drugs known to be more effective.

CHAPTER VII

CONCLUSIONS

DDS is highly inhibitory to M. kansasii. About 20 times as much is needed to inhibit M. smegmatis 607 equally. When DDS inhibition is tested on agar, there is a very narrow range of concentrations between that which exerts no inhibition, and that which totally prevents colony formation. End-points are sharper when single cell inocula are used. There is a lower IC50 when DDS inhibition is studied in liquid culture than when it is measured on agar. The MIC in either situation increases with the period of incubation. There is a very wide range of concentrations over which DDS slows the growth of M. kansasii in liquid culture, whether stationary or shaken. The concentration of DDS that prevents growth is dependent on the number of cells inoculated. With very small inocula, DDS in concentrations greater than 100-fold the IC50 is bactericidal. Exposure of bacilli to DDS results in a delay in the appearance of colonies when the exposed bacilli are inoculated onto drug-free agar. The length of the delay is dependent on the duration of exposure to drug and the concentration of drug to which the bacilli were exposed. Resistant mutants were encountered only rarely and exhibited only a low degree of resistance.

DDS is stable for many weeks in medium stored at either 4° or 37°C. M. kansasii, grown in the presence of DDS, accumulates the drug against a concentration gradient, but neither produces MADDS nor reduces the concentration in the medium significantly.

PABA reverses the inhibition of DDS for both strains of *Mycobacteria* when the two drugs are present in approximately equal amounts. Partial reversal was shown when the concentration of PABA was 1/80 that of the DDS. However, PABA itself inhibits both strains. The growth rate of *M. kansasii* was slowed by 10 µg/ml PABA. This inhibition was neither increased nor decreased by 0.8 µg/ml DDS, which alone produced significantly more inhibition.

DDS produces the requisite post-exposure inhibition and can be considered a candidate for intermittent chemotherapy. Mechanisms for termination of post-exposure inhibition *in vitro* include diffusion of the drug away from the bacillus, and dilution of the amount of drug per bacillus by cellular division that occurs in the presence of drug, although at an inhibited rate.

To the limited extent to which inter-species comparisons are valid, this study confirms *in vitro* the following information derived in the mouse foot-pad with *M. leprae*: inhibition by very low concentrations of DDS, inhibition of a form which prolongs the time before end-point is reached, an initial lag before inhibition is expressed, continuing inhibition following the termination of drug contact, the rare occurrence of resistant mutants, antagonism of DDS inhibition by PABA, and inhibition by PABA.

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APPENDIX I

ABBREVIATIONS

AFB	Acid Fast Bacilli
BCG	A strain of *M. bovis* of reduced virulence
BI	Bacterial Index
BSA	Bovine Serum Albumin
BSS	Balanced Salt Solution
CFU	Colony Forming Unit
DDS	4,4'-diaminodiphenyl sulfone, Dapsone
GU	Glycerol Urea medium
IC50	Inhibitory Concentration at 50% inhibition.
INH	isonicotinyl hydrazide, isoniazid
L-J	Lowenstein-Jensen medium
MADDS	monoacetyldapsone
MI	Morphological Index
MIC	Minimal Inhibitory Concentration
MPN	Most Probable Number
O.D.	Optical Density
PABA	para-aminobenzoic acid
PAS	para-aminosalicylate
S.D.	Standard Deviation

APPENDIX II

SOURCES OF PRODUCTS

BSA, Armour Laboratories, Kankakee, Illinois

Circle Slides, Bellco Glass, Vineland, New Jersey

Coleman Instruments, Inc., Maywood, Illinois

Corn Meal Agar Base, Difco Laboratories Inc., Detroit, Michigan

DDS, K&K Laboratories, Hollywood, California

Dubos Agar Base, Dubos broth base, Dubos medium albumin, Dubos oleic acid albumin supplement, Difco Laboratories Inc., Detroit, Mich.

Farrand spectrofluorometer, Model 242, Farrand Optical Co., Ind., New York.

Glycerol, Nutritional Biochemicals Co., Cleveland, Ohio. Glycerol from several other chemical supply houses was tried, but proved to be inhibitory.

Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, New Jersey

Hand Tally, Homs, Model Tm-4 Counter. The Veeder-Root counter was tried, but had too much resistance to thumb action, causing cramping of hand muscles if more than a couple of hundred colonies had to be counted in one day.

Hanks' BSS, Grand Island Biological Co., Berkeley, California.

Klett-Summerson Photoelectric Colorimeter, Model 880-3, Klett Manufacturing Co., Ind, New York

L-J medium, Hyland Laboratories, Los Angeles, California

Microscope with apochromatic objective

PABA, General Biochemicals, Chagrin Falls, Ohio

Petri Dishes, Falcon Plastics, Los Angeles, California

Resazurin, total dye content 72%, Allied Chemical, New York

Sonifier$^{(R)}$ Cell Disruptor Model W140 D, Heat Systems--Ultrasonics Inc. Plainview, Long Island, New York

Variable speed mixer, Vortex Junior mixer, Scientific Industries, Inc., Queens Village, New York.

APPENDIX III

SOURCES OF *MYCOBACTERIA*

Norman E. Morrison, Johns Hopkins University-Leonard Wood Memorial Leprosy Research Laboratory, Department of Pathobiology, School of Hygiene and Public Health, Baltimore, Maryland. *M. smegmatis* 607.

S.R.Pattyn, Department of Bacteriology, Prince Leopold Institute for Tropical Medicine, Antwerp, Belgium. *M. kansasii*. 3 strains. The one used in the major experiments is his number 915.

Lawrence G. Wayne, Veterans Administration Hospital, Long Beach, California. Many strains of many species. All the strains that were susceptible to DDS other than the above are from this source, including one *M. kansasii*, two *M. gordonae*, one *M. gastri*, and one *M. terrae*.