Studies in Mice of the Action of DDS Against *Mycobacterium leprae*[1]

C. C. Shepard[2]

The method generally used for the study of the activity of drugs against *Mycobacterium leprae* in the mouse foot pad system has been one where the drug is given throughout the infection, beginning the day the mice are infected. The infecting inoculum of *M. leprae* is quite small; in our laboratory it is 5,000 bacilli, so that unless the bacilli multiply there are so few that usually none is encountered during the standard counting procedure of bacilli in suspensions of foot pad tissues. The untreated controls develop about 2×10^6 *M. leprae* per foot pad and this corresponds to about 100 bacilli in the counting procedure. Thus the difference between control mice and mice treated with a completely inhibitory drug, such as DDS, is quite distinct.

Using this approach, we carried out a titration of the activity of DDS ([13]), and were surprised to find that even the lowest amount of DDS tested, 0.00001 per cent, was completely inhibitory.

Other strains of *M. leprae* are now being tested, (([10]) and unpublished results). So far, five other strains have been found not to be inhibited by 0.00001 per cent DDS in the diet. To date nine strains from untreated patients have been tested against 0.0001 per cent DDS in the diet and all have been completely inhibited. Thus 0.0001 per cent DDS in the diet seems to represent the usual level of sensitivity of strains of *M. leprae* from untreated patients.

This intake of DDS does not produce levels of DDS in the blood or tissues that are measurable by the usual (Bratton-Marshall)([1]) procedure; so estimates of the concentration have been made by extrapolation from higher dosages. At measurable levels the blood levels are directly proportional to the percentage of DDS in the diet, and when 0.01 per cent is given the concentration of "free" (ethyl acetate-extractable) sulfone is 1-3 μgm./ml. whole blood ([12, 13]). A concentration of 1-3 μgm./ml. whole blood is produced in man by intake of the standard amounts of 50 to 100 mgm./day in adults. Thus the minimal inhibitory concentration of DDS appears to be about 0.01 to 0.03 μgm./ml. whole blood, or about 1/100 the amount produced in man by standard dosages. Several assumptions were made in arriving at this estimate. One is that the metabolism and excretion of the drug are the same at low levels as at high levels and that the rates of these processes are proportional to drug concentration. Another is that the proportion of drug bound to blood proteins is the same at high and low levels. Still another is that the concentration of drug in the environment of the bacilli is the same as it is in blood. There is evidence supporting these assumptions, but they need complete investigation.

The sensitivity of *M. leprae* has been compared to the reported sensitivities of several other microorganisms, especially in infections in mice ([13]). *M. leprae* appears to be about three times as sensitive as *Plasmodium berghei* ([16]) and about 50-100 times as sensitive as group A streptococci ([4]). In the past the assumption has sometimes been made that the sensitivity of *M. leprae* to DDS is about the same as that of tubercle bacilli, for which the minimal inhibitory concentration is on the order of 5-10

μgm./ml. Since such concentrations were not attained by doses that were clinically effective in leprosy it was assumed that DDS did not act directly against *M. leprae*, and other explanations have been invoked, e.g., that DDS owes its activity to an ability to stabilize lysosomes and that it is the destruction of lysosomes that is responsible for the disease manifestations, or that *M. leprae* produces a metabolite of DDS that is much more active than the parent compound. The finding that the growth of *M. leprae* is in fact inhibited by very small doses of DDS makes such elaborate hypotheses unnecessary, and allows us to assume for the present that the simple hypothesis of direct antibacterial activity by DDS is correct. Presumably the mechanism of action would be an interference with folate biosynthesis, by a substitution of DDS for para-aminobenzoic acid (PABA).

Knowing the minimal effective dosage of DDS against *M. leprae* one can test for antagonism against DDS by PABA. In an experiment that we have carried out (Table 1) the highest subtoxic dose of PABA was tested against the minimal effective dose of DDS, and the antibacterial effect of DDS was partially antagonized. With a smaller amount of PABA the inhibition of DDS was less distinct. With tenfold higher concentrations of DDS, there was no interference with its activity. Although it seems clear that PABA did antagonize DDS, it is difficult to say whether the inhibition was competitive. The amount of PABA in the blood of the mice receiving 1.0 per cent in the diet was 3-4 μgm./ml. (measured by the procedure for "total" sulfone, using PABA as standard). The amount of DDS in the blood of mice receiving 0.0001 per cent had been estimated as 0.01 to 0.03 μgm./ml., so that the highest molecular ratio of PABA to DDS that was tested was apparently about 1:400.

A facet of the sensitivity of *M. leprae* to DDS is the activity of repository sulfones in mice ([10]). These compounds, which had

TABLE 1. *Effect of* p-aminobenzoic acid *(PABA) on the action of DDS against* M. leprae. *The mice were infected with 5,000* M. leprae *and put in cages with the diets indicated on day 0. Counts of acid-fast bacteria (AFB), made on pools of 4 mice from different control groups, showed the following growth curve:* $<1.5 \times 10^4$ *at 60 days,* 3.0×10^4 *at 101 days,* 4.2×10^5 *at 132 days,* 4.7×10^5 *at 168 days, and* 1.4×10^6 *at 196 days. Counts of AFB were then carried out on pools of mice from each group.*

Mouse number	Addition to diet		Harvest (AFB/mouse)	
	DDS (%)	PABA (%)	199 days[a]	234 days[b]
1–20	—	—	7.2×10^5	4.1×10^6
21–40	0.0001	—	5×10^4	4×10^4
41–60	0.0001	1.0	2.7×10^5	1.1×10^5
61–80	0.0001	0.01	6×10^4	1.3×10^5
81–100	—	—	1.4×10^6	—
101–130	—	1.0	7.2×10^5	2.4×10^6
131–160	—	0.01	9.6×10^5	2.5×10^6
161–180	—	—	7.7×10^6	2.2×10^6
181–200	0.001	—	$<1 \times 10^4$	$<6 \times 10^3$
201–220	0.001	1.0	$<1 \times 10^4$	$<7 \times 10^3$
221–240	0.001	0.01	$<1 \times 10^4$	$<7 \times 10^3$
241–260	—	—		

[a] Four mice per pool.
[b] Six to eight mice per pool.

been developed for possible usefulness as malaria preventatives, are insoluble in water and in their suspending vehicle. Their activity is presumed to arise from the slow release by tissue enzymes of DDS or other active metabolites. The repository we have worked with the most is 4,4'-diacetyl-diaminodiphenyl sulfone (DADDS); it is able to suppress growth of *M. leprae* in mice when given in doses as low as 1.5 to 6 mgm./kgm. every two months.

Another implication of the great sensitivity of *M. leprae* to DDS is that contamination with DDS may influence experimental and therapeutic results. Thus in animal experiments one must take precautions against carry-over of DDS in the diet-mixing machine and during feeding. In human therapy with sulfones ordinarily administered in doses of a gram or more a day (e.g., Promin, Sulphetrone, Promizole, Promacetin), the presence of a few tenths of a per cent or more of DDS in the commercial product would probably be enough to account for therapeutic activity. Lowe [5] has reported the presence of such concentrations of DDS in Sulphetrone and Promin.

The results described so far were obtained with a design in which the drug has been given throughout the experiment. This type of experiment does not differentiate between bacteriostatic and bactericidal effects. We are now trying a kinetic method in which the drug is administered for a limited period beginning in the lag phase or early logarithmic phase. By this method one observes the growth curves in the various groups and estimates the amount of delay in the growth curve in a treated group as compared to the control groups. If the delay in growth is limited to the period of administration, it is assumed that a drug is bacteriostatic only. If the delay is more prolonged, bactericidal activity is considered, and an estimate of the maximal amount of killing that could have occurred is made by assuming that the extra delay arises from the necessity for new bacterial growth to replace the fraction killed by drug. Similar experiments involving exposure to drug for a limited time have been used to study the effect of chemotherapeutic agents on bacteria growing *in vitro* [7].

In our first kinetic experiment with *M. leprae* [11] the drugs were administered for 60 days during the second and third months after infection. Counts of *M. leprae* were carried out in the various groups when the bacterial growth in the controls had reached plateau levels, and again three months later. This was, in fact, too infrequent a schedule of counts (in current experiments a more frequent schedule is being used). Nevertheless the experiment showed the following:

1. Streptomycin and thiambutosine (DPT, Ciba-1906) caused bacteriostasis without bactericidal effect during the period of drug administration.

2. Neither 0.01 per cent isoniazid (INH) nor 0.6 per cent para-aminosalicylic acid (PAS) had any antibacterial effect.

3. DDS as 0.1, or 0.01 per cent, in the diet killed part of the bacilli (it was estimated that the killing did not exceed about 80%).

4. The combination of either streptomycin or thiambutosine with 0.01 per cent DDS was no more effective than DDS alone.

5. INH or PAS partially antagonized the antibacterial effect of DDS. This is an unusually large amount of new information to be gained from one experiment with *M. leprae* and attests to the utility of this method. The advantage, of course, comes from the ability of the method to differentiate between varying degrees of antibacterial effect.

The experiment had been planned with the hope that some of the combinations of drugs with DDS would be distinctly bactericidal. However, this hope was not realized. Current experiments are concerned with DDS alone and seek to study the growth curves in more detail, with harvests starting at the end of the period of drug administration and continuing until the plateau phase is reached. The variables under study are the concentration of DDS and the duration of its administration. An early finding is that the time required for DDS to stop growth of *M. leprae* is dependent on the level of the drug; with 0.0001 per cent DDS, the minimal effective dose,

about one month was needed to bring the growth to a halt.

Before going on to discuss some of the steps of the immediate future in research on sulfones and *M. leprae,* I need to mention two developments that are being covered by other speakers in this symposium. First is the important finding of Rees and co-workers ([8]) that sulfone resistance does occur, as demonstrated unequivocally in mice. The strains they studied were isolated from patients with long histories of unsuccessful treatment with DDS. The circumstances were such that a rough calculation could be made that sulfone resistance was quite rare under the conditions in that leprosarium, viz., about one such occurrence in a thousand patients. With Levy we have confirmed Pettit and Rees by the demonstration of DDS resistance in two patients in the United States (unpublished observations). These were patients who had been treated for some years at Carville. Also during routine screening of certain strains being maintained in mice in our laboratory we found one strain resistant to 0.0001 per cent DDS in the diet (tests not completed yet at higher dosages) from a patient said to have been treated with Promin to bacterial negativity during two previous admissions to a leprosarium. At the time of the strain isolation he was entering Carville in his second relapse after having had little or no treatment on the outside, and he had not yet received treatment during that relapse. Thus the *M. leprae* had apparently grown up in the absence of sulfones, indicating that sulfone resistance in this strain was a stable characteristic of *M. leprae* in man. The strain was put through 12 passages in mice over a period of seven and a half years with a total multiplication of 7.8×10^{32}-fold before being tested for drug sensitivity; this demonstrates further that sulfone resistance can be a stable characteristic, or at least that, if back-mutation to sulfone sensitivity does occur, it does not enable the organism to grow any more rapidly in mice. One would have hoped for evidence that sulfone resistance decreased the biologic fitness of *M. leprae.*

The other development is the new method of Glazko ([15]) for determining sulfones at nanogram levels. The Bratton-Marshall procedure ([1]) allows the measurement of sulfones down to the level of about 0.2 μgm./ml. Since the minimal inhibitory concentration of DDS is suspected to be a few hundredths of a μgm./ml., what is needed is a method that is capable of measuring a few thousandths of a microgram/ml., and that is what Glazko's method seems capable of doing.

Our measurements of "free" DDS in mouse blood and human blood are based on extraction from mildly alkaline blood into ethyl acetate. In the estimation of the minimal inhibitory concentration of DDS it is assumed that a good share of the ethyl acetate-extractable sulfone is the parent compound. Actually it would not affect our considerations much if a derivative of DDS was also present in both species, provided it was about as active as DDS; presumably the mono-acetylated and mono-glucuronated DDS might be such compounds. However, our considerations of the kinds of derivatives of DDS that might be present are not based on exact knowledge of the metabolism of DDS, since the metabolites present in blood and urine have not been completely identified, and 20 to 30 per cent of the intake is usually not accounted for. Also the knowledge of metabolic pathways of DDS that has been acquired in the past needs to be checked at low levels of intake.

A related piece of information that is badly needed is knowledge of the persistence rate of DDS (or other active compounds) in blood and tissue of man. Usually this is expressed as a half-life. Smith ([14]) reported the half-life of "free" DDS in blood to be three days. Chatterjee and Poddar ([2]) reported the half-life of S-35-labelled DDS in blood to be 1.6 days. There are several reports that "traces" of DDS may be detected by the Bratton-Marshall procedure several weeks after the cessation of DDS or other sulfones, indicating a very long persistence. However, the reliability of "traces" of color in the Bratton-Marshall procedure is not very great, and such findings need to be checked with more appropriate procedures.

An experiment with one human volunteer is shown in Table 2, which records the blood and urine sulfone concentrations after the ingestion of 100 mgm. DDS (1.23 mgm./kgm. body weight). The method of determination of sulfone in urine was that of Ellard ([3]), in which the amount of sulfone extractable into ethyl acetate at neutral reaction is determined without hydrolysis and with hydrolysis in 1N HCl in a boiling water bath or at room temperature. It is assumed that all of the conjugated DDS is hydrolyzed by hot acid, so that extraction then allows measurement of total DDS. Columns 3 and 4 thus represent the daily output of total DDS (free + conjugated). The logarithms of these values fall nicely on a straight line, indicating that daily excretion decreased with a half-life of 1.3 days and that there was no difference in the excretion mechanism at high and low levels of DDS in blood. The last column represents free DDS in urine. Following the interpretation of Ellard, the values in the next to the last column represent more faithfully the free DDS at the time of excretion by the kidneys and before the nonenzymatic formation of N-glucuronides in the bladder or after collection of the urine. Again the relative constancy of this percentage indicates that the excretion mechanism is the same at high and low levels of DDS in blood. The decrease in blood values is consistent with a half-life also of 1.3 days, and extrapolation indicates that the concentration of 0.03 μgm./ml. blood would be reached in about eight days.

Obviously such experiments need to be repeated in more persons, in order to learn how great the variation is among individuals; blood concentrations should now be determined directly by Glazko's new procedure ([15]). The information gained would be essential to the rational design of a dosage schedule of DDS.

In recent years there have been several clinical trials of lower dosage of DDS in leprosy. Most of these have taken the form of less frequent administration of DDS,

TABLE 2. *Concentrations of sulfone in blood and urine after ingestion of 100 mgm. DDS by a human volunteer. The urine specimens were 24-hour collections made up to 3,000 ml. The sulfone procedure for urine was that of Ellard ([3]); 2 ml. urine was treated with acid as indicated, neutralized, buffered to pH 7.0, and extracted with ethyl acetate (Et-Ac).*

Time after DDS intake	"Free" sulfone in blood (μgm./ml.)	Sulfone in urine. Et-Ac extractable after			
		1N HCl, 100°		1N HCl, 23° (%)	No HCl (%)
		(μgm./ml.)	(mgm./24 hr)		
3 hrs	1.2				
1 day	0.8	11.44	34.32	44	21
2 "	0.4	7.96	23.88	58	39
3 "	0.3	4.02	12.06	59	36
4 "	<0.2	2.66	7.98	50	33
5 "	"	1.38	4.14	51	34
6 "	"	0.92	2.76	51	24
7 "	"	0.42	1.26		
8 "	"	0.12 (0.21)[a]	0.36 (0.63)[a]		
9 "	"	0.08 (0.18)[a]	0.24 (0.54)[a]		
10 "	"	<0.1			
		TOTAL:	87.00 (87.57)		

[a] The values in parentheses were determined on 5 ml. sample of urine.

sometimes on inspiration from the experience in tuberculosis. The primary drugs in tuberculosis, INH and streptomycin, do not remain long in the blood and tissues following administration, and it has been concluded from experimental and clinical results that much of the bacterial killing is associated with peak concentrations of drug rather than the duration of time the concentrations remain above minimal inhibitory concentration ([7]). It now seems likely that, in the case of *M. leprae* and DDS, killing is much slower, whether expressed in hours or in generation times. Also DDS is a slowly excreted drug and levels above the minimal inhibitory concentraton are maintained for long periods; the results shown in Table 2 would indicate that following the administration of 100 mgm. DDS, blood levels above the minimal inhibitory concentration are maintained for about one week.

Helpful information may come from clinical experience with the repository sulfone DADDS. In its first clinical trial as a repository, DADDS is being compared to DDS in collaboration with Leonard Wood Memorial personnel in Cebu, Philippines. The dosage being used is 225 mgm. DADDS every 77 days. In the course of this study bloods have been collected for sulfone determinations from all patients, and 24-hour urines from several of them. As expected, patients receiving DADDS have blood concentrations of sulfone that are not detectable by the Bratton-Marshall procedure; the excretion of total sulfones in their urine has averaged 1.63 mgm./24 hours, and the level of sulfones has not varied much during the period between injections of DADDS. The control patients, who have received full dosage of DDS, had blood concentrations of 2-3 μgm. free DDS/ml. blood; the excretion of total sulfones in the urine has averaged 46.2 mgm./24 hours. On a simple proportionality basis this would indicate that the blood concentrations · in the DADDS patients remained at about 0.07 μgm. free DDS/ml.

In the near future one can expect to see more clinical trials of low dosages of DDS and repository sulfones. These will usually be designed to find whether these new regimens are as effective as full dosage of DDS. Frequently the critical measurement will be the change in morphologic index or solid ratio in the skin smears, and there will also be observations of the bacterial index in skin smears, and of the clinical changes, including ENL. Also, because of the possible appearance of sulfone-resistant *M. leprae*, certain observations should be made to define this phenomenon as closely as possible. Pettit and Rees' study ([8]) indicated that sulfone resistance appeared in about 1 in 1,000 patients for whom standard dosages of DDS by mouth had been prescribed. (Incidentally, on the basis of 10^{11} *M. leprae* per patient ([9]), with 1 per cent viable bacilli, this would correspond to an incidence of sulfone resistance of 1 in 10^{12} bacilli.) Lowe ([6]) followed a group of more than 100 patients on standard amounts of DDS for many years and found that all responded, a finding that also indicates the incidence of resistance to microgram levels of DDS at less than 1 in 100 patients. It is possible that resistance to a lower concentration is more frequent, and the development of resistance to microgram levels of DDS may require several mutations in the presence o f radically changing amounts of DDS. If this is so, it may be that full sulfone resistance usually appears in the course of irregular treatment, and it will be important to distinguish this background from the one where DDS concentrations are at a low but constant level. To clarify this issue it would be essential to have regular measurements of blood sulfone (or urinary excretion of sulfone) in every patient, as a control on ingestion and absorption of the drug in that patient. Also provision should be made for the isolation in mice of the purportedly resistant *M. leprae*, so that the resistance can be authenticated and its level measured.

SUMMARY

Using the experimental design in which the drug is administered to mice from the beginning of the experiment, we have tested nine strains of *Mycobacterium leprae* from untreated patients against 0.0001 per cent DDS in the diet and found all

nine to be sensitive. Six strains have been tested against 0.00001 per cent DDS and only one has been found sensitive. From these results it is estimated that the minimal inhibitory concentration of DDS for *M. leprae* is about 0.02 μg/ml., or about 1/100 the amount produced in human beings by dosages of 50 to 100 mgm./day. *M. leprae* is thus more sensitive to DDS than other microorganisms.

When PABA was administered at a level of 1.0 per cent in the diet it partially antagonized the antileprosy effect of 0.0001 per cent DDS in the diet.

Using a kinetic method for testing drugs we have been able to distinguish bactericidal from pure bacteriostatic effects. The kinetic method was used to test the bactericidal effect of several combinations of drugs with DDS, but none was found any more effective than DDS alone.

The great sensitivity of *M. leprae* to DDS suggests that leprosy might be treated as effectively by spaced administration of DDS or by injection of one of the new repository sulfones. In clinical trials along these lines several precautionary observations probably ought to be made and these are discussed.

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DISCUSSION

Dr. Prabhakaran. Were sulfonamides tried in leprosy and discarded because they were found ineffective?

Dr. Shepard. Published data on long-acting sulfonamides show that they have some effectiveness.

Dr. Brown. It seems possible that a sulfone could get into the mycobacterial cell more easily than a water-soluble sulfonamide. This might account for the finding that para-aminobenzoic acid is relatively ineffective in reversing the sulfone effect. The water-soluble sulfonamides resemble PABA in that they too might fail to penetrate the heavy lipid layer of the leprosy bacillus.

Dr. Skinsnes. There is a footnote in Cochrane's text book in which he makes a statement regarding difficulties he had with sulfapyridine and some other sulfonamides in the treatment of leprosy. In several instances there was increased incidence of ENL. The reasons for this have never been explained. I have recently seen sulfapyridine given to a lepromatous patient, and the effect was as described in Cochrane's textbook. This particular patient, a woman, has experienced an unusual amount of ENL and other reactions for a period of about four years. She was entirely on sulfapyridine to begin with.

Dr. Shepard. Dr. Rees also has tried long-acting sulfonamides in the mouse foot pad model and Dr. Gaugas recently has reported that several of these were active.

Dr. Karat. We have made a small trial with 5 mgm. of DDS a day, six days a week, for the last two years. Preliminary observations support Shepard's report of findings in the mice. There are two expected changes. First, changes in the morphologic index (MI) from about 10 down to 0, in from three to five months. Second, a drop in the total bacterial population, as evidenced by drop in bacterial index in skin smears from eight sites made at monthly intervals. In the two years during which these patients were followed, the drop in the MI and bacterial population has been comparable to that following dosages of 100 mgm. per day. In our studies 25 patients with lepromatous leprosy are now on long-acting sulfonamides. Under the conditions of the trial in the initial phases they have shown the same response as patients on 5 or 100 mgm. DDS.

Dr. Shepard. What has been the incidence of ENL in these groups?

Dr. Karat. In the 5 mgm. group the incidence of ENL has been almost the same as in the 100 mgm. group. This is contrary to the experience of other workers. However, we have found that the severity of ENL, and the necrosis of ENL, is much less in the 5 mgm. group, as compared with the 100 mgm. group. We have not been able to reduce the incidence or prevalence of ENL in the lepromatous group by reducing the dose of DDS to 5 mgm. instead of 100 mgm. a day.

Dr. Bushby. I wonder whether either Dr. Shepard or Dr. Thompson knows if, in the fluorometric method, direct fluorometric measurements are made or whether conjugation, or rather diazo reactions, are involved.

Dr. Shepard. It is a direct procedure in which some extraction is involved.

Dr. Bushby. This method might estimate substances we have not previously recognized, such as deaminated ones.

Dr. Shepard. Extraction in an organic solvent is involved.

Dr. Bushby. Therefore even if amino groups were lost, the compounds might still be detected,

Dr. Skinsnes. In the 1950's, in Hong Kong, Dr. Smiley and Dr. Fraser and I also used about 50 mgm. per week in patients who had trouble with ENL. This resulted in greatly diminished severity of ENL, much less interruption of therapy, and favorable progress. I have been watching another patient with lepromatous leprosy in Chicago for the last two years, who was doing fairly well on a higher dosage level, but then developed ENL. He was then put on 50 mgm. per week, and now seems to be progressing equally well. He has had no further acute episodes.

Dr. Shepard. In conclusion I want to add some remarks on the sensitivity of the urine extraction procedure. One can measure 0.1 microgram per ml. of urine if one starts out with a 2 ml. sample. This is based on urine made up to 3,000 ml. in a 24-hour collection. Furthermore, since urine concentrations of DDS are about 10 times the blood concentrations, one can detect the presence of much smaller amounts of DDS in the blood by studying urine.