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Studies on the Determination of the Minimal Inhibitory Concentration of 4,4'-diamino-diphenyl-sulphone (Dapsone, DDS) against *Mycobacterium leprae**

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Plasma DDS concentrations were determined fluorometrically in mice fed continuously with 0.01% of the drug in their diet. They averaged 0.74 μg per ml.

(2) Since it is known that the multiplication of *Myco. leprae* in the mouse footpad system is inhibited by feeding 0.0001% DDS but not by 0.00001% DDS in the diet, it was concluded that the MIC of DDS against *Myco. leprae* must be less than 0.01 μg per ml.

(3) The half-life of DDS in the mouse was determined after intraperitoneal dosage and found to be 2.7 h after dosage with DDS at 10 mg per kg and 3.7 h after 50 mg per kg.

(4) DDS serum concentrations in patients who were being successfully treated with 1 mg of DDS a day averaged 0.018 μg per ml 3 h after daily dosage, and a similar proportion of the drug was excreted in the urine as was found with doses some 50 to 300 times as large.

(5) The significance of these findings is discussed both in the relation to the treatment of leprosy with DDS, and to the use of the mouse footpad system in predicting the likely efficacy of other potential antileprosy compounds.

Introduction

When diamino-diphenyl-sulphone (DDS) was first introduced for the treatment of leprosy, the doses used were chosen empirically since at that time no laboratory method was available for measuring the sensitivity of *Mycobacterium leprae* to

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the drug. Daily doses of up to 100 mg DDS by mouth or twice weekly doses of 300 mg by injection were found to be reasonably well tolerated by most patients, and to be therapeutically effective. For these reasons weekly dosage with 600 mg of DDS gradually became accepted as the standard treatment.

The discovery by Shepard (1960) that bacteria from leprosy patients multiply to a limited extent in the footpads of mice made it possible to test experimentally the effectiveness of drugs in inhibiting the multiplication of strains of *Myco. leprae* from previously untreated patients. The activity of DDS in preventing multiplication of the organisms in the mouse footpad system was first demonstrated by Shepard and Chang (1962). In this first study, DDS was fed at a dietary concentration of 0.1%. Two years later they showed that its antileprosy activity was still maintained when it was given in a dietary concentration of 0.01% (Shepard and Chang, 1964). These findings were confirmed by Rees (1965) and Pattyn and Royackers (1965). Since these initial studies, there have been several investigations to determine the minimal effective dose of DDS necessary to inhibit the multiplication of *Myco. leprae* in the mouse footpad system. The results that have been obtained are summarized in Table 1. All the strains of *Myco. leprae* were inhibited by feeding DDS at a dietary concentration of 0.0001%, and with but two exceptions, they were not inhibited by a dietary concentration of DDS of 0.00001%. The minimal effective dose of DDS against *Myco. leprae* is therefore somewhere between these two limits.

TABLE I

Activity of DDS against M. leprae *in the mouse foot-pad system*

% DDS in the diet				
0.0001[a]	0.00005	0.000025 and 0.00003	0.00001	References
A (1)[b]		A (1)	A (1)	Shepard *et al.* (1966)
A (2)			IA (4)	Shepard (1967*a*)
A (9)			IA (5)	Shepard (1967*b*)
A (2)			IA (2)	Rees (1967*a*)
A (2)			A (1) IA (1)	Rees (1967*b*)
A (11)				Shepard *et al.* (1969)
A (12)	A (1)	A (1)	IA (5)	Shepard (1969)[c]
A (25)			IA (24) A (1)	Rees (1970), current data.

A = Active, inhibition of multiplication.
IA = Inactive.
[a] = All concentrations above 0.0001% active.
[b] = Number of strains in parenthesis.
[c] = Personal communication.

Since it is still not possible to cultivate *Myco. leprae in vitro,* the minimal inhibitory concentration (MIC) of DDS against the organism cannot be measured directly. However, it can be estimated by measuring the concentration of DDS in the serum or plasma of mice fed with the minimal effective dose of the drug required to inhibit the multiplication of *Myco. leprae.* Since DDS is a relatively

non-polar, uncharged, lipid-soluble compound, it would be expected that it would readily penetrate cell membranes so that tissue concentrations would parallel the concentrations of the drug in the blood. Its rapid absorption in man (Glazko *et al.*, 1968) and its penetration into the tissues in mice (Shepard and Chang, 1964; Rees, 1967*b*) and in man (Chatterjee and Poddar, 1957) are in accord with these assumptions.

In this study we have measured the plasma concentrations of DDS using a new fluorometric method which is considerably more sensitive and potentially more specific than the colorimetric methods previously employed. This method has also been used to determine two potential metabolites of DDS, namely, *N*-acetyl-DDS (MADDS) and *N N'*-diacetyl-DDS (DADDS, acedapsone). The earlier colorimetric studies indicated that the concentration of DDS in the serum of mice receiving 0.0001% of the drug (the minimal effective dose) was approximately 0.01 μg per ml, or only about one-hundredth of the minimal serum concentrations achieved in man after daily dosage with 100 mg of the drug. A pilot clinical trial was therefore undertaken to establish whether a dose of as little as 1 mg of DDS a day was also effective in the treatment of lepromatous leprosy (Waters *et al.*, 1968). This clinical trial has now been completed and has established the effectiveness of this dose of DDS (Waters and Rees, 1971). In order to facilitate a direct comparison between the results achieved in the clinical study and the previous experimental studies using the mouse footpad system, we also measured the concentrations of DDS in the plasma of the patients participating in this clinical trial, using the same fluorometric method employed to determine the MIC of the drug against *Myco. leprae* in the mouse.

Methods

MEASUREMENT OF THE CONCENTRATIONS OF DDS, MADDS AND DADDS IN THE PLASMA OF MICE

Plasma was obtained between 09.00 and 11.00 h from a total of 13 female P-strain albino mice who had been fed continuously with 0.01% DDS in the diet for periods ranging from 6 to 10 months. It was estimated that continuous feeding with DDS should result in steady-state plasma concentrations of the drug being achieved within one day. The mice were exsanguinated individually, following cardiac puncture under ether anaesthesia. Plasma was also collected from 2 groups each of 15 mice which had been dosed with DDS, 10 and 50 mg per kg body-weight respectively, by intraperitoneal injection. Each mouse received the drug dissolved in 1 ml of ethanol/polyethylene glycol 300/0.85% sodium chloride (1 : 3 : 6 parts by volume), and the mice, in groups of 3, were killed 1, 2, 4, 6 and 24 h, respectively, after dosage. Initial experiments demonstrated that the vehicle was well tolerated by the mice, as were doses of DDS of up to 50 mg per kg in the vehicle; doses of 100 and 200 mg per kg in the vehicle, however, were lethal.

Approximately 0.3 ml of plasma was obtained from each mouse. This was diluted to 3 ml with water and the concentrations of DDS, MADDS and DADDS determined by the fluorometric method of Ellard and Gammon (1969). Standards, which were extracted in duplicate, consisted of 3 ml of water, normal mouse plasma, aqueous 1 μg per ml DDS, MADDS, and DADDS, respectively. For these studies, DDS was extracted into 3 ml of 1.2 N-HCl, rather than 2 ml as in the original method.

The plasma DDS concentrations in mice which had been dosed intraperitoneally with DDS, 50 mg per kg, were also determined, using a modification of the Bratton and Marshall (1939) colorimetric procedure (Ellard *et al.*, 1970). Fifteen minutes after reaction the absorption spectrum was scanned in a Unicam SP 800 spectrophotometer and the concentration of DDS calculated from the extinction at 570 mμ. Standards which were extracted in duplicate consisted of 3 ml of water, normal mouse plasma, and aqueous 10 μg per ml DDS, respectively.

MEASUREMENT OF THE SERUM CONCENTRATION AND URINARY EXCRETION OF DDS IN PATIENTS PARTICIPATING IN A THERAPEUTIC TRIAL OF 1 mg OF DDS DAILY

The design and conduct of the clinical trial has been described elsewhere by Waters and Rees (1971). Serum samples and 24-h urine collections were both obtained from the patients immediately before commencing treatment and thereafter alternating every 3 weeks during treatment. Blood samples were taken 3 h after dosage with DDS during the 6th, 12th, and 18th weeks of treatment. Glazko *et al.* (1968) have shown that DDS is rapidly absorbed in man after oral dosage and that peak serum concentrations occur 2 to 4 h after dosage. During this period DDS serum concentrations are almost constant, and thereafter fall slowly, with a half-life of about 21 h.

Urine collections were made during the 3rd, 9th, 15th and 18th weeks of treatment. Each 24-h urine collection was diluted to 2.4 litres and an aliquot was preserved by the addition of 1% (by volume) glacial acetic acid (giving a pH of between 3 and 4). The urine and serum samples were stored at −20° C until despatch on ice by air from Malaya to England for analysis. It had previously been shown that DDS was stable in serum for at least 6 months when stored at −20° C.

The concentration of DDS in the serum and urine samples was determined using a modification of the method of Ellard and Gammon (1969). Serum standards, which were extracted in triplicate, consisted of 3 ml of water, normal serum, and normal serum containing 0.01, 0.1 and 1.0 μg per ml DDS, respectively. Urine standards, a 24-h collection which were extracted in quadruplicate, consisted of 10 ml of water, normal urine (from a healthy subject, G.A.E.) diluted to 2.4 l after the addition of 24 ml of glacial acetic acid, and normal urine containing 0.01, 0.1 and 1.0 μg per ml DDS, respectively.

Next, 3 ml of serum was extracted by shaking with 8 ml of ethyl acetate and 1 ml of M sodium citrate in a stoppered centrifuge tube; 10-ml aliquots of urine were extracted by shaking with 10 ml of ethyl acetate, 2 ml of M sodium citrate and 8 g of ammonium sulphate. Thereafter the serum and urine extracts were treated identically. Of the ethyl acetate extract 6 ml was washed by shaking with 1 ml of 0.1 N sodium hydroxide and 5 ml of the washed extract further washed by shaking with 1 ml of 0.1 N hydrochloric acid; 4 ml of this washed ethyl acetate extract was then extracted by shaking with 3 ml of 1.2 N hydrochloric acid. Duplicate 1-ml aliquots of the 1.2 N hydrochloric acid extract were then pipetted into small centrifuge tubes. To the first, 0.1 ml 1% (w/v) aqueous sodium nitrite was added to destroy the fluorescence of any DDS present, presumably through diazotization of the aromatic amino groups, and 5 min later the nitrite was destroyed by the addition of 0.1 ml of 10% (w/v) ammonium sulphamate. Two ml M sodium citrate was then added and the mixture extracted by shaking

with 2 ml of ethyl acetate. The second 1-ml aliquot was extracted by shaking with 2 ml of M sodium citrate and 2 ml of ethyl acetate without the prior addition of nitrite and sulphamate. Each ethyl acetate extract was then dried by shaking with 0.5 g of anhydrous sodium sulphate.

The concentration of DDS was determined by measuring the fluorescence of the second ethyl acetate extract at 298/345 mμ and subtracting from it the fluorescence of the first nitrite-treated extract. In this way each sample also provided its own blank. The fluorescence of the extracts was also measured at 298/420 mμ and 295/324 mμ, respectively.

Results

PLASMA DDS CONCENTRATIONS IN MICE

The results obtained are summarized in Tables 2, 3 and 4. That the fluorometric method employed specifically measured unchanged DDS in the plasma of the mice was apparent for 4 reasons: (1) plasma from normal mice gave

TABLE 2

Concentrations of DDS in the plasma of mice fed continuously with 0.01% DDS in the diet

Experiment	Concentrations of DDS (μg/ml)	Fluorescent characteristics	
		Ratio[a] 420/345 mμ	Ratio[a] 324/345 mμ
1	0.73, 0.39	0.26 (0.24)[b]	0.39 (0.40)
2	0.65, 0.99, 1.19, 1.00, 0.90	0.22 (0.26)	0.34 (0.36)
3	0.35, 0.57, 0.69, 0.24, 0.54, 1.38	0.33 (0.32)	0.32 (0.35)
Mean[c]	0.74 ± 0.10	–	–

[a] Ratio of fluorescence at 298/420 mμ and 295/324 mμ, respectively, compared with that at 298/345 mμ.
[b] Ratios for 1 μg/ml aqueous DDS standards in brackets.
[c] Mean ± standard deviation of mean.

TABLE 3

Concentrations of DDS in the plasma of mice after 10 mg/kg DDS intraperitoneally

Time (h)	Plasma DDS concentrations (μg/ml)		Fluorescent characteristics	
	Individual	Mean[a]	Ratio[b] 420/345 mμ	Ratio[b] 324/345 mμ
1	6.59, 5.83, 4.59	5.61	0.32	0.32
2	2.45, 5.41, 3.69	3.66	0.35	0.34
4	2.51, 2.72, 2.46	2.56	0.33	0.34
6	1.30, 1.42, 1.65	1.45	0.34	0.35
24	0.18, <0.10, <0.10	–	0.32	0.68
1 μg/ml aqueous DDS standard (3 ml)			0.32	0.35

[a] Geometric means.
[b] Ratio of fluorescence at 298/420 mμ and 295/324 mμ, respectively, compared with that at 298/345 mμ.

TABLE 4

Concentrations of DDS in the plasma of mice after 50 mg/kg DDS intraperitoneally

Time (h)	Plasma DDS concentrations (μg/ml)		Flourescent characteristics	
	Individual	Mean[a]	Ratio[b] 420/345 mμ	Ratio[b] 324/345 mμ
1	18.6, 24.6, 29.8	23.9	0.34	0.36
2	15.3, 22.1, 19.2	18.7	0.32	0.34
4	16.0, 14.5, 12.5	14.3	0.33	0.33
6	7.77, 9.92, 9.76	9.10	0.33	0.35
24	<0.10, 0.43, 0.15	—	0.63	0.47
1 μg/ml aqueous DDS standard (3 ml)			0.32	0.35

[a] Geometric means.

[b] Ratio of fluorescence at 298/420 mμ and 295/324 mμ, respectively, compared with that at 298/345 mμ.

extracts whose fluorescence did not differ significantly from those of the aqueous blanks, so that the fluorescence of the extracts from the treated mice must have been due to compounds derived from DDS; (2) the fluorescent characteristics of the extracts from the treated mice were very similar to those of the DDS standards and unlike those of MADDS, which also extracts to a small extent in this solvent system; (3) when duplicate 1-ml aliquots of the 1.2 N hydrochloric acid extracts were heated for 1 h at 100°C prior to subsequent extraction, they gave ethyl acetate extracts whose fluorescent characteristics were identical to those of DDS, and whose absolute fluorescence was only about 6% greater than those of the unheated extracts; and (4) specificity studies showed that DDS-N-glucuronide does not extract into ethyl acetate and it is known that DADDS does not extract into 1.2 N hydrochloric acid (Ellard and Gammon, 1969).

From the variation in the fluorescence of replicate extracts from the aqueous blank and normal mouse plasma, it was concluded that the method could be used to measure concentrations as low as about 0.1 μg per ml DDS in 0.3 ml of plasma.

The plasma concentrations found in mice which were being continuously fed with 0.01% DDS in their diet are summarized in Table 2. These concentrations ranged from 0.2 to 1.4 μg per ml and averaged 0.74 μg per ml.

The concentrations of DDS in the plasma of mice after intraperitoneal dosage with 10 and 50 mg of DDS per kg, respectively, are summarized in Tables 3 and 4 and illustrated in Fig. 1. The half-life of DDS in the mouse was calculated from regression analysis of the logarithms of the DDS plasma concentrations at 1, 2, 4, and 6 h after dosage. These analyses showed that the plasma concentrations of DDS fell exponentially throughout this period at rates equivalent to a half-life of 2.7 h after a dose of DDS of 10 mg per kg and 3.7 h after 50 mg per kg. The difference between these half-lives was significant ($P < 0.0001$). If the fall in DDS concentrations had continued exponentially at each dose, the concentrations at 24 h would be expected to be 0.01 μg per ml and 0.33 μg per ml, after doses of 10 and 50 mg per kg, respectively. The results obtained are in reasonable agreement with these predictions and do not suggest any major change in the rate of fall of the plasma concentrations of DDS at these later times.

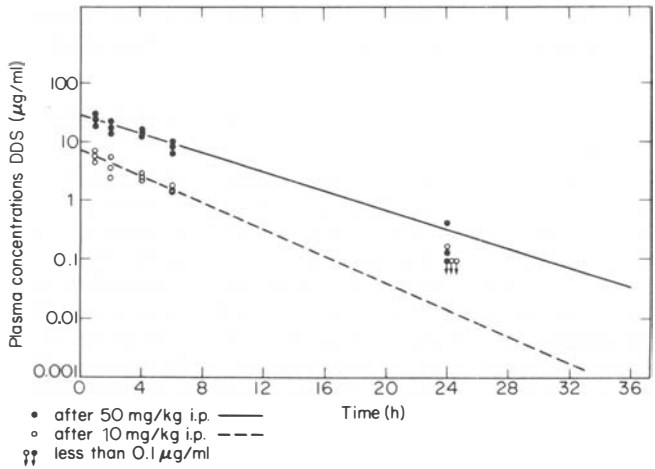

Fig. 1. Concentrations of DDS in mice after dosage with 10 and 50 mg per kg.

From the regression analyses it was concluded that the replicate errors were equivalent to about ±18% after 50 mg per kg DDS and ±24% after 10 mg per kg DDS. Since these coefficients of variation are composite error terms that include errors in dosage, timing, and the fluorometric DDS determinations, as well as variations between individual mice, it is probable that the individual mice did not vary greatly in the rates at which they metabolized or excreted DDS.

PLASMA MADDS AND DADDS CONCENTRATIONS IN MICE

Convincing evidence for the presence of MADDS in the plasma of mice was not obtained. The fluorometric MADDS determinations and the estimated amounts of acid-hydrolysable DDS extracted into 1.2 N hydrochloric acid indicated that after intraperitoneal dosage with 50 mg of DDS per kg the MADDS concentrations could not have exceeded a sixth of the concomitant plasma DDS concentrations. DADDS was not detected in these plasma samples and it was concluded that the concentrations of DADDS could not have exceeded 0.2 μg per ml or 1% of the concomitant DDS concentrations.

PLASMA DDS CONCENTRATIONS IN MICE DETERMINED BY THE COLORIMETRIC METHOD

The reacted extracts showed similar extinction profiles to that of DDS with its characteristic hump and maximal absorption at 570 mμ. This is presumably because both the aromatic amino groups of DDS can be diazotized and coupled with N-1-naphthyl-ethylene-diamine. The ratio of the extinctions at 570 mμ to those at 545 mμ averaged 1.05 for the extracts from the DDS-treated mice compared with 1.08 for the DDS standard. By contrast, MADDS and most other aromatic monoamines show a near symmetrical extinction profile, with a peak absorption at about 545 mμ (for MADDS the ratio of the extinction at 570 mμ to that at 545 mμ was about 0.75). Although the absorption spectra of the reacted extracts were thus very similar to that of DDS, the absolute concentrations of DDS determined colorimetrically in this way were about 25% greater than those determined using the more specific fluorometric method. A comparison of the

results obtained by the two methods indicated that the sensitivity of the fluorometric method was at least 5 times greater than that of the colorimetric method.

SERUM CONCENTRATIONS AND URINARY EXCRETION OF DDS IN PATIENTS BEING TREATED WITH 1 mg OF DDS DAILY

Regression analysis showed that the fluorescence of the extracts from both the serum and urine standards included in the analyses of the serum and urine samples from the patients treated with 1 mg of DDS per day, was directly proportional to the concentration of DDS over the range 0.01 to 1.0 μg per ml. In each case the error of the method in this concentration range was estimated at ± 0.010 μg per ml. The fluorescence of the extracts of the normal serum and urine used to prepare the standards, and of the nitrite-treated extracts from the DDS standards, was not significantly greater than the fluorescence of the aqueous blank extracts.

The fluorescence of the extracts of the pre-treatment serum samples from the patients did not differ significantly from that from the aqueous blank, but the extracts from the serum samples obtained 3 h after dosage with 1 mg of DDS showed significant fluorescence at 298/345 mμ ($P < 0.001$). The mean fluorescence of these extracts was equivalent to a concentration of DDS of about 0.018 μg per ml. However, the error of the method (equivalent to ±0.010 μg per ml DDS) was such that it was not possible to measure individual DDS serum concentrations. Nevertheless, it was calculated that the serum concentrations of DDS in the patients never exceeded 0.05 μg per ml. Since tablets of DDS that might have been available locally to the patients would have contained at least 50 mg of DDS, it was concluded that surreptitious self-medication with alternative supplies of DDS could not have occurred in any of the 4-day periods prior to the serum collections. The fluorescent characteristics of the compound extracted from the serum of these patients was, considering the inaccuracies of the method at these low concentrations, fairly similar to that of DDS (Table 5).

Unlike the ethyl acetate extracts from the urine used for setting up the DDS standards, the extracts from the patients' pre-treatment urine samples showed

TABLE 5

Concentrations of DDS in human serum 3 h after daily dosage with 1 mg DDS

Serum DDS concentrations (μg/ml)	Fluorescent characteristics	
	Ratio[a] 420/345 mμ	Ratio[a] 324/345 mμ
0.018 ± 0.003[b]	0.40	0.37
0.1 μg/ml standard	0.25	0.41
1 μg/ml standard	0.26	0.41
0.4 – 4.1[c]	0.28	0.41
1 μg/ml standard	0.27	0.39

[a] Ratio of fluorescence at 298/420 mμ and 295/324 mμ, respectively, compared with that at 298/345 mμ.

[b] Mean ± standard deviation of mean.

[c] For comparison, from a group of dermatitis herpetiformis patients being continuously treated with 50-300 mg of DDS per day, analysed in a similar way (Ellard and Gammon, 1969).

significant fluorescence. Part of this fluorescence was due to compounds that were destroyed by treatment with nitrous acid. The fluorescence characteristics of these extracts were markedly different from those of DDS (Table 6), but were similar to those from urine samples collected from other patients in the Sungei Buloh leprosarium who were either temporarily untreated or else being treated with drugs other than DDS. The fluorescence of the extracts of the urines obtained during daily treatment with 1 mg of DDS was significantly greater than that of the pre-treatment extracts ($P = 0.01$) and the increase in fluorescence at 298/345 mμ was equivalent to the urinary excretion of about 0.18 mg of DDS per day. Further, the fluorescence characteristics of the treatment extracts were more like that of DDS than were those of the pre-treatment extracts. The fluorescence ratios of the compound excreted during daily dosage with 1 mg of DDS were calculated for each patient by subtracting the fluorescence of the pre-treatment extract from that of each treatment extract. It will be seen from Table 6 that the mean fluorescence ratios were similar to those of DDS. For comparison, the results that were obtained from a group of patients with dermatitis herpetiformis who were being treated with some 50 to 300 times this dose of DDS (Ellard and Gammon, 1969) are also shown.

Discussion

The concentrations of DDS found in the serum or plasma of mice, together with determinations of its half-life in the mouse, found in this study are compared in Table 7 with the results obtained by other workers. The mean concentration of DDS in the plasma of mice which had been continuously fed with 0.01% DDS in the diet averaged 0.74 μg per ml. This is in good agreement with the results obtained by Shepard *et al.* (1966) and Bushby and Rees (Rees, 1967*b*) using colorimetric methods for the determination of DDS. In the latter study serum was adjusted to pH 1 and heated for 1 h at 100° C. After cooling the pH was adjusted to 8 and DDS extracted into methyl isobutyl ketone and thence into 2 N hydrochloric acid, and determined by a modification of the Bratton and Marshall (1939) procedure (Bushby, personal communication). Tissues were incubated with papain at 56° C overnight and then treated in the same way as serum. Our results are also similar to those obtained by Glazko and Shepard (personal communication) and Ozawa *et al.* (1971) using either the fluorometric method of Glazko *et al.* (1968) or that employed in this study (Ellard and Gammon, 1969).

After intraperitoneal dosage, the half-life of DDS in the mouse was found to be 2.7 h after dosage with 10 mg per kg, and 3.7 h after 50 mg per kg. These results are similar to those obtained by Gordon *et al.* (1970) after dosing mice intraperitoneally with DDS, 1 mg per kg, and by Ozawa *et al.* (1971) after terminating continuous dosage with 0.01% DDS in the diet.

The concentrations of DDS at time zero after intraperitoneal DDS dosage with 10 and 50 mg per kg were calculated by extrapolation as being 6.8 and 28 μg per ml, respectively. The fact that the plasma concentrations of DDS fell exponentially from the earliest time of collection (1 h) indicated the absorption of DDS from the peritoneum, and its subsequent distribution throughout the body was extremely rapid. Its volume of distribution in the mouse would appear to be about 1.6 times the body weight. Somewhat similar results were obtained by Francis and Spinks (1950), using an oral dose of DDS of 100 mg per kg, and Gordon *et al.* (1970) after an intraperitoneal dose of 1 mg per kg.

TABLE 6

Urinary excretion of DDS (μg per ml)

Urine Samples	Dose (mg DDS/day)	Apparent concentration (μg DDS/ml)	Excretion (mg/day)	Excretion (% dose)	Fluorescence characteristics	
					Ratio[a] 420/345 mμ	Ratio[a] 324/345 mμ
Pre-treatment	nil	0.043 ± 0.013[b]	–	–	0.42	0.95
Treatment	1	0.117 ± 0.012	–	–	0.32	0.67
Treatment	1	increment 0.074 ± 0.012	0.178 ± 0.029	18	0.23	0.51
Standards	–	(0.1-1.0)	–	–	0.23	0.41
Treatment[c]	50-300	(0.8-13.5)	(2-32)	17	0.26	0.41
Standards	–	(1.0)	–	–	0.25	0.39

[a] Ratio of fluorescence at 298/420 mμ and 295/324 mμ, respectively, compared with that at 298/345 mμ.
[b] Mean ± standard deviation of mean.
[c] For comparison, from a group of dermatitis herpetiformis patients analysed in a similar way (Ellard and Gammon, 1969).

TABLE 7

Concentrations of DDS in the serum, plasma or blood of mice

% DDS in the diet													
0.2	0.1	0.05	0.025 and 0.03	0.016	0.01	0.006	0.003	0.001	0.0001	Single dose (mg/kg)	Half-life DDS (h)	Method[a]	Reference
				3.9[b]						248	5-6[b]	C	Titus and Bernstein (1949)
		9.1										C	Francis and Spinks (1950)
										100	9-10[c]	C	Francis and Spinks (1950)
	15.3											C	Francis (1953)
22.1[d]	19.0	2.4	5.2		2.6							C	Shepard and Chang (1964)
			3.6[e]		0.93[e]		0.49[e]	0.14[e]				C	Shepard *et al.* (1966)
	12.5		3.3		0.89	0.55		0.15				C	Rees (1967b)
					1.00			0.063	0.007			F	Glazko and Shepard (1969)[h]
										1[f]	2.6	F	Gordon *et al.* (1970)
					0.79			0.093	0.011			F	Ozawa *et al.* (1971)
											3.6-5.0[g]	F	Ozawa *et al.* (1971)
					0.74							F	This study
										10[f]	2.7	F	This study
										50[f]	3.7	F	This study

[a] Methods: C = colorimetric; F = fluorometric.
[b] Calculated from the data illustrated in Figs 1 and 2 of Titus and Bernstein (1949).
[c] Calculated from the data illustrated in Fig. 6 of Francis and Spinks (1950).
[d] Food consumption reduced.
[e] Means calculated from data in Table 1 of Shepard *et al.* (1966) weighted with number of mice whose blood was pooled for analysis.
[f] Administered intraperitoneally.
[g] After terminating continuous dosage with 0.01% DDS in the diet.
[h] Personal communication.

The acute toxicity of DDS was manifest within a few minutes after intraperitoneal dosage with 100 mg per kg. This would have been expected to result in plasma concentrations of DDS of about 50 μg per ml. Boyer (1951) has previously found the LD^{50} of orally administered DDS in the mouse to be 600 mg per kg, although it is possible that under some conditions of oral dosage DDS may be rather less rapidly absorbed (Titus and Bernstein, 1949). Nevertheless, these latter workers did obtain peak blood DDS concentrations of about 100 μg per ml 7 h after dosage with 248 mg per kg without reporting the occurrence of acute toxicity. It should be noted that Francis (1953) has obtained evidence that suggests that old, large mice are less likely to develop nervous symptoms due to DDS on account of its poorer penetration into their brains than are young, small mice.

Mice tend to eat continuously throughout the day and night. The mice used in these studies had an average weight of 25 g, and consumed 5 g of diet a day (Rees, 1967*a*). If it were assumed that the mice eat continuously at the same rate throughout the day and night, then the formula derived by Wagner (1969) for a "one compartment open model" with a constant rate of infusion can be used to calculate the "steady state" DDS plasma concentrations that should be attained. This formula states that:

$$C = \frac{R}{V \cdot K}$$

where R is the rate of infusion, V the volume of distribution and K the first order rate constant for the overall loss of DDS in the mouse. Thus feeding mice with 0.01% DDS in the diet, with a drug half-life of 2.7 to 3.7 h would be expected to result in an average steady state plasma concentration of DDS of 0.20 to 0.28 μg per ml. Although this calculated value is only approximately one-third of the actual value found experimentally, it does at least provide an explanation for the fact that continuous dosage with 0.001% DDS in the diet of the mouse (equivalent to about 2 mg of DDS per kg per day) gives rise to DDS plasma concentrations that are less than a tenth of those achieved with an equivalent dose in man (100 mg per day) in whom the half-life of the drug is about 21 h. This discrepancy had previously been noted by Rees (1967*a*).

The results obtained by Shepard *et al.* (1966), Bushby and Rees (Rees, 1967*b*), Glazko and Shepard (personal communication) and Ozawa *et al.* (1971) (Table 7) indicate that the plasma concentrations of DDS in the mouse are approximately proportional to dose over the dosage range 0.0001 to 0.01% DDS in the diet. Thus since the minimal effective dose of DDS against *Myco. leprae* in the mouse is less than 0.0001% in the diet (Table 1), the average minimal inhibitory concentration of DDS against *Myco. leprae* in the mouse footpad system must be less than 0.01 μg per ml. It should be emphasized that these calculations are based on the average concentrations of DDS expected in mice during the day. The actual concentrations probably vary above and below these values throughout the day according to the feeding habits of the mice. Such factors are probably a major cause of the variation between the results from different mice shown in Table 2.

The fact that MADDS concentrations in the mouse were less than one-sixth, and DADDS concentrations less than one-hundredth, of the concomitant DDS concentrations is in accord with the results of Ozawa *et al.* (1971) and other workers. Thus Glazko and Shepard (personal communication) found similar "total acid-hydrolysable" and "free" DDS plasma concentrations in mice after

dosage with DDS, and Baukema, Chang and Glazko (personal communication) found only traces of MADDS in the urine after dosage with 7 mg per kg of ^{35}S-labelled DDS. Furthermore, Gordon *et al.* (1970) were unable to detect MADDS in the plasma of mice which had been dosed intraperitoneally with either DDS at 1.0 mg per kg or MADDS at 1.2 mg per kg, and concluded that the dose of MADDS had been entirely deacetylated to form DDS.

The serum concentrations of DDS in the patients who were being treated with 1 mg of DDS daily averaged 0.018 μg per ml 3 h after dosage. By comparison, using the same fluorometric method, we have previously found a DDS concentration of 1.8 μg per ml in the serum of a patient with dermatitis herpetiformis 4 h after repeated daily dosage with 100 mg of DDS (unpublished results). Glazko *et al.* (1968), using their fundamentally similar fluorometric method, found peak DDS plasma concentrations of about 1.2 μg per ml in a group of 5 volunteers after a single dose of 100 mg of DDS. Thereafter the DDS plasma concentrations fell exponentially, with half-values averaging 20.6 h. Similar results have been obtained by Gelber *et al.* (1971) using the fluorometric method of Peters *et al.* (1970). On the basis of these data it was calculated that daily dosage with 100 mg of DDS would eventually lead to peak DDS plasma concentrations of 2.2 μg per ml, and trough values of 1.0 μg per ml. These results suggest therefore that in man, serum or plasma concentrations after daily dosage with 1 mg of DDS are almost exactly one-hundredth of those obtained after daily dosage with 100 mg DDS.

The urinary excretion of DDS by the patients who were being treated with 1 mg of DDS daily averaged 0.18 mg, or 18% of the dose. Since these urine samples had been preserved by the addition of acetic acid, it may be assumed that a considerable proportion of any DDS-N-glucuronide that had been excreted would have already been converted to DDS before analysis. The results may be compared with an excretion of 17% DDS by a group of subjects who were being treated with 50 to 300 mg of DDS a day, or with the excretion of about 10% DDS and 15% DDS-N-glucuronide after single 100-mg doses of the drug (Ellard and Gammon, 1969; Gelber *et al.*, 1971).

Considering both the DDS serum concentrations of the patients who were being treated with 1 mg of the drug daily, and the amount of the unchanged drug they excreted in the urine, it would appear that the absorption, metabolism and excretion of DDS when given at this dosage is similar to that found with doses 50 to 300 times as great. This would suggest that the trough values of the DDS serum concentrations in the patients being treated with 1 mg of the drug daily probably averaged about 0.010 μg per ml.

In the mouse footpad system, the multiplication of *Myco. leprae* was prevented by dosing the animals with 0.0001% DDS in the diet, which it was calculated gave rise to serum/plasma concentrations of DDS that averaged less than 0.01 μg per ml throughout the day. The fact that patients with lepromatous leprosy were successfully treated with 1 mg of DDS daily (Waters and Rees, 1971), a dose that resulted in serum concentrations of the drug ranging from about 0.01 to 0.02 μg per ml, suggests that with this drug the results obtained in the mouse footpad system can be used directly to predict the efficacy of novel DDS regimens.

Treatment with 1 mg of DDS daily appeared, from the fall in the morphological indices, to be as effective as 50 mg of the drug twice-weekly (Pearson and Pettit, 1969), or 300 mg of the drug given twice-weekly by intramuscular injection (Waters, 1963; Waters and Pettit, 1965) in rendering

Myco. leprae non-viable in lepromatous patients, at least during an initial period of 4½ months (Waters and Rees, 1971). The rate of fall of the morphological indices was also similar to that achieved with clofazimine (Pettit *et al.*, 1967). The evidence at present available indicates that at concentrations near their minimal inhibitory concentrations (MIC's) the sulphonamides are bacteriostatic drugs *in vitro* and that they show bactericidal activity only at much higher concentrations. It would therefore seem probable that at all the doses of DDS used in these clinical trials, which have ranged from an average of 1 to 100 mg per day, the drug has primarily acted by preventing the multiplication of *Myco. leprae* in the patients, and that the fall in the morphological indices was due to killing by the cell-mediated defence mechanisms of the patients. The fact that in many series only about 35% of the bacilli in untreated lepromatous patients are viable, provides direct evidence of the capacity of the host to kill *Myco. leprae.* Furthermore when leprosy is treated with rifampicin, a drug known to show bactericidal activity against other organisms, the rate of fall of the morphological index is more rapid than that encountered with DDS or clofazimine (Rees *et al.*, 1970).

The results obtained in this study are also in accord with the findings of Shepard *et al.* (1968), who demonstrated the therapeutic effectiveness of intramuscular injections of 225 mg of DADDS given once every 77 days, and showed that such treatment resulted in the urinary excretion of amounts of acid-hydrolysable DDS equivalent to daily release in the tissues of about 2.5 mg of DDS.

It should be emphasized, however, that all these clinical studies have been of too short a duration to detect the potential emergence of DDS-resistant strains, which would, by analogy with tuberculosis, be expected to be apparent only after several years' treatment.

In the studies carried out using the mouse footpad system, compounds are generally tested for their potential antileprosy activity by giving them in the diet. If it is true that the mice feed continuously throughout the day and night so that approximately constant tissue and plasma concentrations of the compounds are achieved, then the MIC's determined by measuring these steady state concentrations at the minimal effective doses of the drugs in question are obviously analogous to MIC's determined *in vitro* against cultivatable pathogens.

In the treatment of tuberculosis there is, on the whole, a good correlation between the activities of drugs *in vitro* and *in vivo.* Thus, those compounds whose toxicity is low enough for them to be given in doses that result in plasma concentrations that are many times their MIC's *in vitro* are generally more powerful antituberculosis drugs than those whose tolerable plasma concentrations in man are only 2 to 4 times greater than their MIC's against *Myco. tuberculosis.*

The MIC's of 2 other drugs, the long-acting sulphonamides sulphadimethoxine and sulphadoxine, have also been determined recently against *Myco. leprae,* using the mouse footpad system (Ellard *et al.*, 1970). Like DDS (half-life about 21 h) these drugs also have relatively long half-lives in man (about 36 h and 1 week respectively). It should therefore be possible to predict with a reasonable degree of certainty the likely efficacy of all 3 compounds in the treatment of human leprosy. The plasma concentrations of sulphadimethoxine and sulphadoxine that are achieved in man at the dosages normally employed are about 4 to 15 times their MIC's against *Myco. leprae* as determined in the mouse footpad system (Ellard *et al.* 1970). Initial treatment with these drugs should therefore be

expected to produce results similar to that achieved with doses of 1 to 100 mg of DDS per day. However, Rees (1967*a*) has suggested that the effectiveness of long-term treatment with doses of DDS averaging about 100 mg per day, doses which produce concentrations of DDS in the body of over 100 times the MIC of DDS against *Myco. leprae,* could be due to the rarity of natural mutants of *Myco. leprae* with these degrees of resistance. If this hypothesis is true, long-term chemotherapy with much lower doses of DDS, or with sulphadimethoxine and sulphadoxine may be less successful, and the possibility of relapse due to the appearance of drug-resistant mutants of *Myco. leprae* after several years' treatment must be entertained.

The fact that the half-life of DDS in the mouse (about 3 h) is much shorter than that in man (about 21 h) may have important implications for experimental studies designed to investigate the potential effectiveness of intermittent DDS chemotherapy in the treatment of human leprosy. Rees (1967*a*) has already demonstrated the effectiveness of once-weekly dosage with 0.01% DDS in preventing the multiplication of *Myco. leprae* in the mouse. Studies on the effectiveness of the treatment of experimental and human tuberculosis with isoniazid given intermittently have shown that the extent to which doses can be spaced out before chemotherapy breaks down is directly related to the half-life of isoniazid in the body. Thus in man, when the interval between the isoniazid doses was extended from 3 or 4 days to 7 days, treatment began to break down in the rapid inactivators (isoniazid half-life about 1 h) but not in the slow inactivators of the drug with an isoniazid half-life of about 3 h (Tuberculosis Chemotherapy Centre, Madras, 1970). Isoniazid has a similar half-life in the guinea-pig to that in human rapid inactivators, and in guinea-pigs intermittent chemotherapy with an equivalent dose of isoniazid also broke down when the interval between the doses was extended from 4 to 8 days (Dickinson *et al.,* 1968). It would therefore seem likely that if a given intermittent regimen was effective in inhibiting the multiplication of *Myco. leprae* in the mouse footpad system, then the corresponding intermittent regimen in man (with same mg per kg dose of DDS) should be at least as effective in the treatment of human leprosy.

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