Further Experience with the Kinetic Method for the Study of Drugs Against *Mycobacterium leprae* in Mice.

Activities of DDS, DFD, Ethionamide, Capreomycin and PAM 1392[1, 2]

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The first results with the kinetic method were recently described ([9]). In the present work the method has been used to study more drugs and to investigate the activity of DDS as it is affected by dosage and duration of administration.

MATERIALS AND METHODS

The general procedure was to follow the growth curves of *M. leprae* in control mice and in mice treated for limited periods. Harvests of the inoculated foot pads (and counts of bacilli) were begun in control groups at about 90 days after infection and in treated groups on the day the drug was stopped. Harvests were then continued at about 45-day intervals until the growth curve was clearly in the plateau phase (near or above 10^6 bacilli per mouse), or until no more mice remained. Details of the technics have been described ([7, 8, 9, 14]).

Capreomycin was kindly supplied through the courtesy of Drs. J. M. McGuire and H. R. Black, Eli Lilly and Company; DFD by Dr. David P. Jacobus, Walter Reed Army Institute for Research, and PAM 1392 by Dr. Paul E. Thompson, Parke Davis and Company. DDS was purchased from K and K Laboratories.

RESULTS

In the first experiment to be described (Fig. 1) the drugs were given for a period of 91 days beginning 73 days after infection. Capreomycin (10 mgm./day), ethionamide (0.2% in the diet) and DFD (0.1%), were compared to DDS (0.1%). Capreomycin only partially suppressed bacterial multiplication, and only while it was being given; as soon as the drug was stopped, multiplication went on at the normal rate. In contrast, ethionamide and DFD delayed the growth more than 300 days, or more than DDS did; whether the difference from DDS is significant is questionable, however, because of the irregularity in appearance of growth when it is delayed that much, as is seen below.

In the experiment depicted in Figures 2 and 3, DDS was studied in several periods of administration and in several dosages. The period was varied (Fig. 2) from 29 days to 119 days with the dosage held constant at 0.01 per cent in the diet (the intake that produces blood and tissue concentrations equivalent to those produced in man by standard dosages of DDS [10, 11]). In general, the longer DDS was given, the longer bacterial growth was delayed. There was some irregularity of the late results, growth seemingly appearing in the mice treated 119 days before it did in those treated 89 days. However, not many bacterial counts could be carried out then, because not many mice had survived that long.

In the same experiment the period of administration was held constant and the dosage varied (Fig. 3) from 0.01 per cent to 0.00001 per cent in the diet (0.0001% is the usual minimal effective dosage in mice with strains from untreated patients [10, 16, 17]). Also one group was given 0.0001 per cent DDS continuously from the day of inoculation for 253 days; the results in this group show that the strain of *M. leprae* was

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[2] The following abbreviations are used throughout: AFB = acid-fast bacteria; DDS = 4,4-diaminodiphenyl sulfone (dapsone); DFD = 4,4'-diformyldiaminodiphenyl sulfone; PAM = 2,4-diamino-6-(3,4-dichlorobenzylamino) -quinazoline.

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fully sensitive to 0.0001 per cent DDS in the diet by the continuous method.

In the mice treated from 104 to 193 days, 0.00001 per cent DDS was without effect on the growth of *M. leprae*. Dosages of 0.0001, 0.001, and 0.01 per cent DDS did, however, stop the growth, at least temporarily, as shown by the harvests between 193 and 300 days. The bacterial population at which they stopped the growth varied with the dosage; apparently the lower dosages took longer to bring growth to a halt. To estimate the bacterial populations at which multiplication had halted, the harvests between 193 and 300 days were averaged arithmetically for each dosage (at low bacterial population the sampling error in individual harvests is large). The logarithms of these averages are 5.02 for 0.0001 per cent, 4.85 for 0.001 per cent, and 4.38 for 0.01 per cent DDS. These bacterial populations amount to 35, 24, and 8 days of growth along the control curve after start of drug in the three respective dosages; these times are used below in the consideration of the amount of growth delay.

After 300 days, bacterial growth first appeared in the mice treated with 0.0001 per cent DDS, and then in the mice treated with 0.01 per cent DDS. (In the mice given 0.001% DDS the last harvests were from single mice on day 473 and 474; the harvests were in the intermediate range so it was not clear whether late bacterial growth had occurred.)

In another experiment (not illustrated) PAM 1392 was fed at a dosage of 0.1 per cent in the diet for a period of 90 days starting 75 days after infection. The harvests in the untreated mice had passed 10^6 by 120 days. The harvests in the mice treated with PAM 1392 were $10^{5.9}$ at 166 days (the day after drug was stopped) and $10^{6.1}$ at 209 days. Thus the drug had no detectable activity against *M. leprae*.

Method of interpretation. The length of the delay in appearance of bacterial growth in a treated group was measured graphically by comparison with the untreated control group (or the average of the control groups) at a position late in the logarithmic phase (Table 1). Earlier in the logarithmic phase the bacterial counts are subject to more sampling variation. For this estimate to be valid the logarithmic portions of the growth curves need to be parallel, and the results in Figures 1-3 show they were within limitations of the accuracy of the bacterial counts. In the earlier work ([9]) the counts had been performed too infrequently to be sure of this point. Examples of slower than normal growth after short exposure to drug have been observed with tubercle bacilli in bacteriologic medium ([1]).

If a treatment is only bacteriostatic while the drug is present, and no bacteria are killed, bacterial growth would take place at a normal rate as soon as the drug disappeared from the tissues, and the length of growth delay would be no greater than the period of drug administration, plus the time required for elimination of drug. If a treatment produces more permanent antibacterial effects, either by killing bacteria or by rendering them temporarily incapable of multiplication, the length of growth delay would be greater.

In making a differentiation between a treatment that is only bacteriostatic while drug is present, and one that produces residual antibacterial effects, an accurate estimate may be needed of the time required for elimination of the drug (more precisely, the time required for the drug concentration in the vicinity of the bacilli to fall below the minimal inhibitory concentration). With rapidly eliminated drugs such as capreomycin or ethionamide, even several-fold errors in the estimation of elmination time are not important. Errors of this magnitude are important with slowly excreted drugs, however, especially when they are administered at intakes many times the minimal effective dosage, as they were in some groups treated with DDS. Direct measurements of DDS in the pertinent concentrations were not available, so the elimination time was estimated from a previous experiment in which the growth of *M. leprae* was observed during discontinuous administration of DDS. In that experiment where 200 mgm./kgm. was injected every ½, 1 or 2 months, the results suggested that the drug persisted three weeks after injection ([10]). In Table 1 the

TABLE 1. *Drugs tested by the kinetic method.*

1. Experiment	2. Drug	3. Dosage[a]	4. Time administered (days)	5. Estimated time for excretion[b] (days)	6. Estimated time of effective exposure to drug (days)	7. Bacterial growth delay[c] (days)	8. Bacteriostatic effect	9. Delay due to bactericidal effect[d] (days)	10. Fraction surviving (%)
A.	DDS	0.1%	91	20	111	283	—	172	0.0076
	DFD	0.1%	91	20	111	>310[e]	—	>199	<0.0016
	Ethionamide	0.2%	91	1	92	>299[e]	—	>207	<0.0010
	Capreomycin	10 mgm.	91	1	92	67	Partial	None	100.
B.	DDS	0.01%	29	15	44	42	Complete	None	100.
			59	15	74	134	—	60	3.58
			89	15	104	228	—	124	0.10
			119	15	134	184	—	50	6.25
		0.00001%	89	0	0	0	None	None	100.
		0.0001%	89[f]	5	59	120	—	61	3.40
		0.001%	89[g]	10	75	243[e]	—	168	0.0090
C.	PAM 1392	0.1%	90	—	0	0	None	None	100.

[a] Percentage in diet, except in case of capreomycin where it is daily subcutaneous dose.
[b] See text.
[c] Estimate for Experiment A is based on the time that growth curves passed $10^{5.6}$ (Fig. 1). That for Experiment B is based on the time curves passed 10^{6} (Figs 2 and 3).
[d] Column 7—Column 6.
[e] On the basis of normal logarithmic multiplication after the last harvest.
[f] Only 54 days after growth had come to a stop.
[g] Only 65 days after growth had come to a stop.

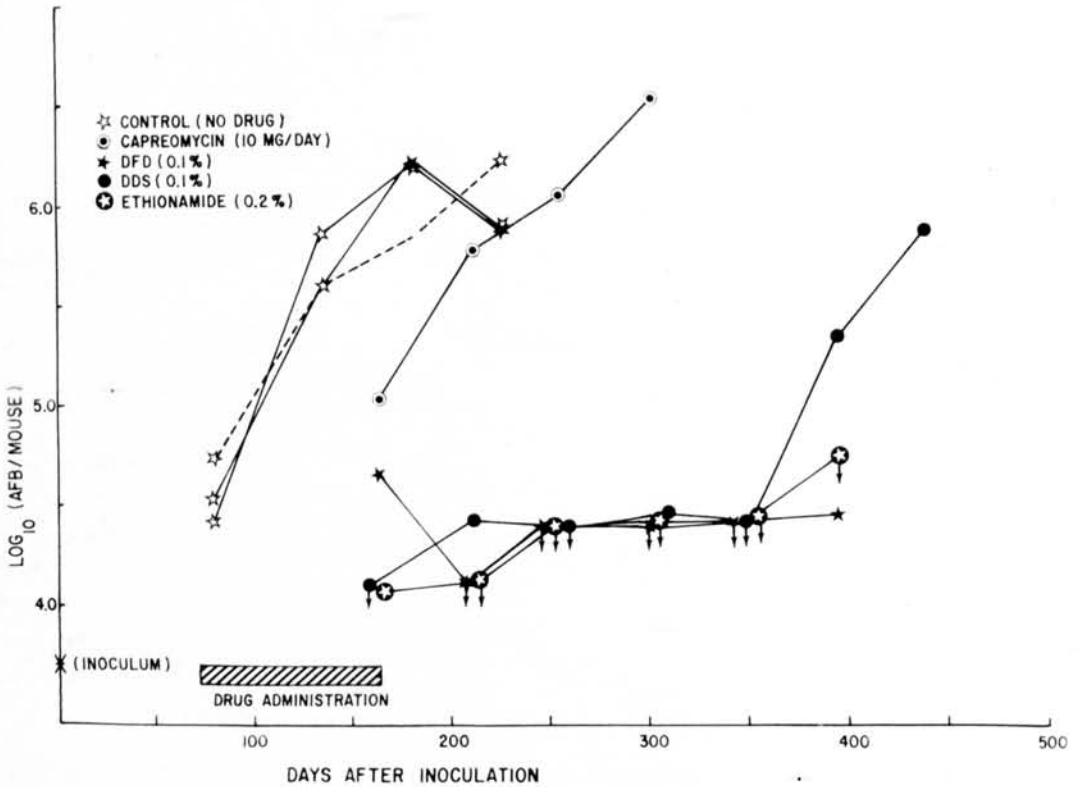

FIG. 1. Comparison of anti-*M. leprae* activity of capreomycin, ethionamide, 4,4′-diaminodiphenyl sulfone (DDS), and 4,4′-diformyldiaminodiphenyl sulfone (DFD). The drugs were given only for a 91-day period beginning 73 days after infection. Capreomycin was injected subcutaneously in a dose of 10 mgm. five days a week; the other drugs were mixed in the unpelleted diet in the amounts shown. The controls and capreomycin mice received unpelleted diet. There were four mice per pool until 226 days, and two mice per pool thereafter. In this and succeeding figures the arrows indicate the maximal estimate of AFB (no AFB were seen during the counting procedure, and the estimate corresponds to the value calculated for one AFB).

elimination time following 0.1 per cent DDS is taken as 20 days, and the times for lower dosages are assumed to be proportional to the logarithms of the dosage and blood level. The estimated elimination time was confirmed in the present study with the results following 0.01 per cent DDS for 29 days, where the assumed time of 15 days fit the data well. The elimination time of DFD, or its active metabolites, is not known and may be longer than the 20 days entered in Table 1.

With 0.001 per cent and 0.0001 per cent DDS (Fig.3) the method of estimating bacterial growth delay due to drug had to be modified, because of the delayed onset of bacteriostasis when the drug was started. In these instances, if the treatment had been merely bacteriostatic in the presence of drug, the growth curve delay would have been shortened by the number of days of treatment required to bring growth to a halt. This adjustment is accomplished in Table 1 by adding this number of days to the direct graphical estimate of growth delay.

Interpretation according to drug. (Table 1) DDS when administered for one month in a dosage of 0.01 per cent was only bacteriostatic while drug was present. This treatment exposed the bacilli to drug for a period estimated to be 44 days, and the growth delay was measured as only 42 days. When 0.01 per cent DDS was admin-

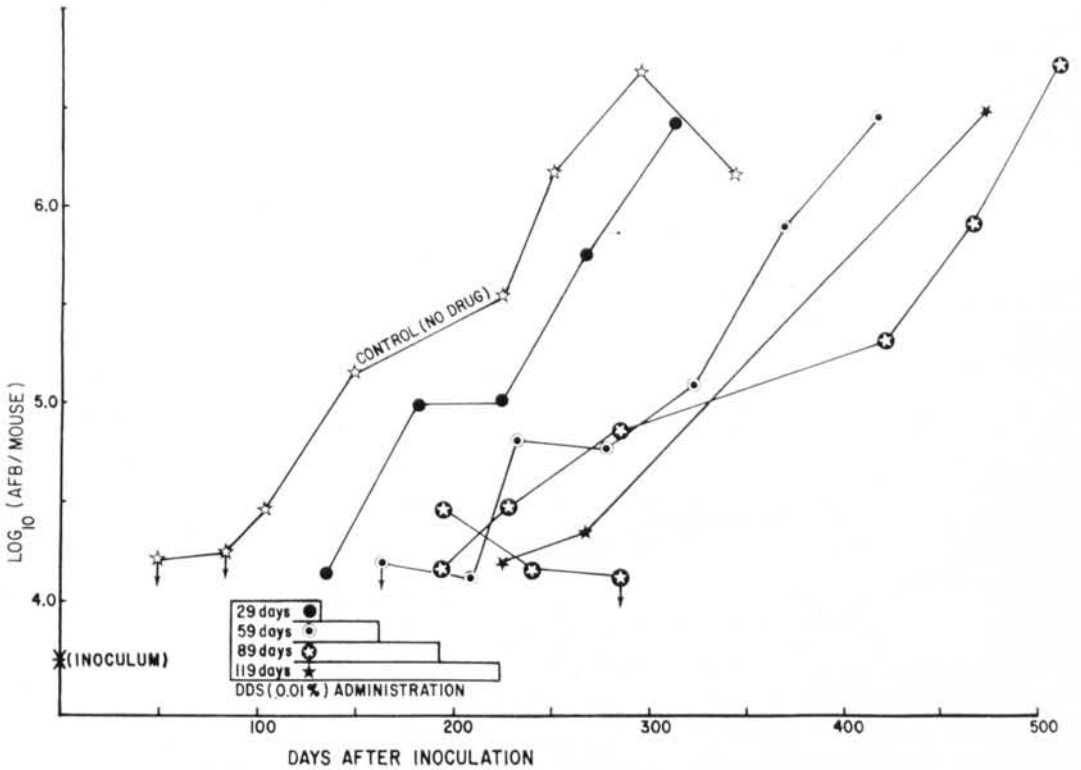

FIG. 2. Anti-*M. leprae* effect of different periods of administration of DDS (0.01% in the diet). DDS was started 104 days after inoculation and continued for the period shown. There were four mice per pool until 342 days, and two to three per pool thereafter.

istered for longer periods, the growth delay was much greater than the period the bacilli were exposed to drug, and the residual effects indicated in column 9 were produced.

The dosage of DDS did not seem to change the effect when allowance was made for the longer time required for lower dosages of drug to bring growth to a halt.

The results with DFD are difficult to interpret with present knowledge. It is converted to DDS *in vitro* by liver homogenates ([3]), but its rate of conversion in the intact mouse is not known.

Ethionamide treatment was as effective as DFD treatment, both preventing growth of *M. leprae* completely within the period of observation. Ethionamide is a rapidly eliminated drug, however, so its prolonged effect would not be attributable to persistence of drug in the tissues.

Capreomycin treatment did not produce complete bacteriostasis while the drug was present and as soon as treatment was stopped the bacteria began to grow at normal logarithmic rates.

DISCUSSION

The continuous method of drug testing cannot discriminate varying degrees of antibacterial activity, whereas the kinetic method reveals that the active drugs fall into two categories: (a) those merely bacteriostatic in the presence of drug and (b) those capable of producing more severe damage to the bacteria so that after the drug has disappeared from the environment the bacteria remain incapable of multiplication, either temporarily or permanently. Clinical therapeutic activity would seem much more likely in the latter category. In the former category of drugs fall streptomycin ([9]) and capreomycin; the latter category DDS, ethionamide, and probably DFD. Thiambutosine, having

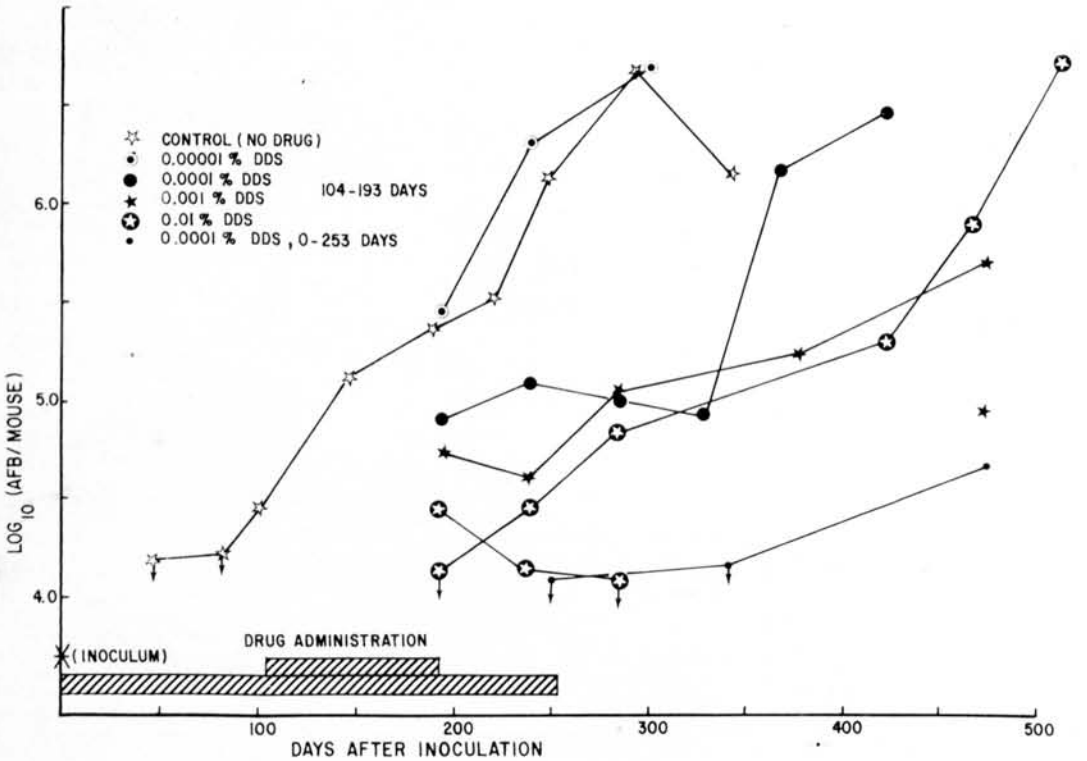

FIG. 3. Anti-*M. leprae* effect of different concentrations of DDS started 104 days after inoculation and continued for 89 days. In addition, one group was given 0.0001% DDS starting on the day of inoculation. The number of mice per pool was usually four until 342 days, and one to three thereafter.

only a short residual effect (9), probably should be consigned to the former group. Inactive drugs by the kinetic method were aminosalicylic acid (PAS), (9), isoniazid (INH) (9), and the quinazoline PAM 1392.

In the preliminary publication (9) the temporary suppression of growth while drug was present was termed bacteriostasis, and the suppression of growth that persisted after the drug had disappeared was termed bactericidal effect. The technic did not, of course, differentiate bactericidal effect from a bacteriostasis that persisted after the drug has disappeared. In the case of bactericidal effect, the amount of growth delay persisting after the disappearance of drug would be the time required for the surviving bacteria to multiply back to the original population. In the absence of bactericidal effect, the growth delay persisting after disappearance of drug would be the time required for the individual bacilli to lose the drug and to recover from the damages caused by the drug.

These different types of residual antibacterial effects have been described for cultivable bacteria in experiments in which survivors were measured by plating them out. Usually there was a mixture of bactericidal activity and residual bacteriostatic effect, that is, both temporary and permanent suppression of colony forming ability was observed. With non acid-fast bacteria Eagle and Musselman (2) found that residual bacteriostatic effects persisted up to eight hours after removal of penicillin (by penicillinase). With *M. fortuitum* Hurwitz *et al.* (4) described delays up to four days in the appearance of colonies after the bacteria had been exposed to streptomycin. Perhaps more pertinent to work with *M. leprae* is the work of Dickinson and Mitchison (1) with *M. tuberculosis*. They

exposed bacilli in growth medium to drug for short periods, replaced the medium with drug-free medium and followed the number of surviving bacterial units by colony counts. With the more active drugs (isoniazid, streptomycin, cycloserine, and ethionamide) they found growth delays of 8-19 days. The delays in the cited studies ([1, 2, 4]) amount roughly to 15 to 30 generation times, a period that would correspond to 6 to 12 months for *M. leprae*.

With *M. leprae*, experiments of this type would have to be carried out by inoculating mice with serial dilutions of the infected tissues of mice that had been treated for various intervals, an approach requiring an impractical amount of effort.

A more feasible method has been the inoculation of single dilutions of tissues in order to learn the length of treatment required to lower bacterial infectivity below detectable limits. One such study was carried out in mice treated continuously with 0.1 per cent DDS ([12]). The infections in the subinoculated mice were not delayed convincingly, as compared to controls, until 88 days of treatment. After this time infectivity was only barely detectable in one specimen. Similar work has been carried out in humans; mice were inoculated with bacilli from skin biopsy specimens taken at intervals from patients entering treatment with DDS ([15]). The infectivity for mice was lost after 90 days of treatment; it was estimated that this corresponded to a loss down to less than 1 per cent of original infectivity. In these studies the stained morphology of bacilli converted from solid to nonsolid, thus affording additional evidence that the drug-induced damage was severe and irreparable ([6, 13]).

In studies *in vitro*, such as those just reviewed, the medium in which the bacilli are suspended affects the results; the medium has to be changed when the drug treatment is stopped, and in most studies also when the bacilli are plated out. That such changes affect the results is shown by observations of "bactericidal" effects that continue after drug is removed ([1, 5]); such observations demonstrate changes in viability that occur in the suspending medium but not in the plating-out medium. With *M. leprae* we are forced to work *in vivo*; the drug is removed by the animal, and in the kinetic method the bacilli are not even disturbed from their intracellular environment. The same is, of course, true of the bacilli in the treated patient.

In column 10 of Table 1 an estimate of the maximum possible bactericidal effect is made by assuming that all of the growth delay persisting after elimination of active drug is caused by death of *M. leprae*, and that none of it is caused by temporary bacteriostasis; in this estimate all of the subsequent appearance of bacteria is assumed to come from multiplication of surviving bacteria at usual logarithmic rates. Our best previous estimate of the logarithmic rate was 12.5 days/generation ([13]), and subsequent experience has been confirmatory. Also confirmatory is the present data; in Figure 1 the rate in the controls between 90 and 135 days averaged 11.9 days, and in Figures 2 and 3 the rate between 221 and 249 days was 13.4 days/generation.

The estimates in column 10 are expressed as the fraction of bacteria surviving at the time drug was eliminated, and the values for DDS-treatment range from 0.01 per cent to 6 per cent when DDS dosage was at least 0.0001 per cent and the time the bacilli were exposed to drug was 60 days or more. These values are compatible with the estimates of bacterial killing in the studies of *M. leprae* in mice ([12]) and in man ([15]), and fail to suggest the presence of significant periods of bacteriostasis persisting after disappearance of DDS.

PAM 1392 is active against *Plasmodium berghei* in mice, *P. cynofologi* and *P. knowlesi* in monkeys and *Trypanosoma cruzi* in tissue cultures and in mice, and hemolytic streptococci *in vitro*. Since the activity against *T. cruzi* is partially reversed by folinic acid, and since the activity against *P. berghei* is synergistic with sulfonamides, the site of action appears to be in the synthetic pathway of folic acid ([18]). PAM 1392 had been tried against *M. leprae* in the hope that, if it were inactive, it might potentiate the activity of DDS. Cycloguanil pamoate, another antimalarial drug which acts in the folic acid pathway

and acts synergistically with DDS against plasmodia, had previously been found inactive against *M. leprae* ([10]),

SUMMARY

Further experience is described with the kinetic method for the study in mice of the activity of drugs against *M. leprae*. In this procedure the drug is administered for a period of 1 to 3 months beginning early in the logarithmic phase of bacillary growth. The method allows differentiation of drug activities causing mere bacteriostasis in the presence of drug, from activities causing residual effects, viz., bactericide and bacteriostasis persisting after the drug has been eliminated.

In one experiment capreomycin (10 mgm., 5 days a week), 0.2 per cent ethionamide in the diet, and 0.1 per cent DFD (4, 4′-diformyldiaminodiphenyl sulfone) were compared with 0.1 per cent DDS (4, 4′-diaminodiphenyl sulfone). Capreomycin was only bacteriostatic, whereas ethionamide and DFD caused residual effects as severe as those caused by DDS. In a second experiment varying concentrations of DDS were given for a fixed period (3 months) and a fixed concentration (0.01% in the diet) was given for varying periods. The lower dosages took longer to stop growth, but otherwise no difference between dosage levels could be distinguished. When the bacteria were exposed to DDS for about 40 days, there was mere bacteriostasis in the presence of drug, but with longer exposure residual antibacterial effects were produced. In a third experiment 2, 4-diamino-6-(3, 4-dichlorobenzylamino)-quinazoline (PAM 1392), a drug with antimalarial and antitrypanosomal activity, was found inactive against *M. leprae*.

RESUMEN

Se describen experiencias ulteriores con el método dinámico para el estudio en ratones de actividad de drogas contra el *M. leprae*. En este procedimiento la droga se administró durante un período de 1 a 3 meses al comienzo de la fase logarítmica del crecimiento bacilar. El método permitió la diferenciación en las actividades de las drogas, entre la acción puramente bacteriostática por presencia y la actividad que produce efectos residuales, tal como efectos bacteriostáticos y bactericidas que persisten después que la droga ha sido eliminada.

En un experimento se compararon la capreomicina (10 mg. durante 5 días a la semana), 0.2 por ciento de etionamida en la dieta y 0.1 por ciento DFD (4-4′-diformildiamino-difenil sulfona) con 0.1 por ciento de DDS (4-4′-diaminodifenil sulfona). La capreomicina fué solo bacteriostática, en tanto que la etionamida y el DFD causaron efectos residuales tan severos como los producidos por el DDS. En un segundo experimente se dieron concentraciones variables de DDS durante un período fijo (3 meses) y a una concentración fija (0.01% en la dieta) durante períodos variables. Las dosis más bajas demoraron más en detener el crecimiento, pero fuera de esto no se observó diferencias entre las diferentes dosis. Cuando las bacterias fueron expuestas al DDS durante alrededor de 40 días, sólo se observó bacteriostasis, pero con exposición más larga se produjeron efectos antibacterianos residuales. En un tercer experimento la 2,4-diamino-6-(3,4-diclorobenzylamino)-quinazolina (PAM-392), una droga con actividad antimalárica y antitripanosómica, no demostró actividad contra el *M. leprae*.

RÉSUMÉ

Description d'une nouvelle expérience utilisant la méthode cinetique pour l'étude de l'activité des medicaments contre *M. leprae* chez les souris. Dans cette technique le medicament est administré au début de la phase de croissance logarithmique du bacille et pendant 1 a 3 mois. Il est ainsi possible de différencier les médicaments donnant une simple bacteriostase au cours de leur administration de aux présentents des effects persistants (par exemple bactériostatiques ou bactericides) aprés leur élimination.

Au cours d'une expérience, les médicaments suivants ont été comparés: capréomycine (10 mgm./jour, 5 jours par semaine), régime alimentaire contenant 0.2% d'éthionamide et 0.1% de DFD (4,4′-diformyldiaminodiphenyl sulfone). La capréomycine s'est montrée seulement bactériostatique tandís que l'éthionamide et la DFP ont présenté des effets résiduels aussi sévères que ceux de la DDS.

Dans une deuxième expérience, des doses variables de DDS ont été administrées pendant une période déterminée (3 mois) et un régime alimentaire contenant 0.01% du même médica-

ment a été donné durant des temps variables. Les doses frables ont mis plus longtemps pour arrêter la multiplication bactérienne, mais c'est la seule difference qui a été observée.

Lorsque les bactéries ont été exposées à la DDS pendant une quarantaine de jours, il a été observé une simple bactériostase en présence du médicament, tandis qu'avec une exposition plus longue, des effets antibactériens persistants ont été mis en évidence.

Dans une troisième experience, la 2,4-diamino-6-(3-4-dichlorobenzylamino)-quinazoline (PAM 1392, qui a une activité dans le paludisme et la trypanosomiase) s'est montrée inactive contre *M. leprae*.

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