THE INFLUENCE OF 4:4'-DIAMINODIPHENYL SULFONE (DDS) ON THE RESPIRATION, REPRODUCTION AND MUTATION OF MYCOBACTERIA [1]

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Although 4:4'-diaminodiphenyl sulfone (DDS) and its derivatives were first employed for the treatment of tuberculosis and have long been used for the treatment of leprosy, comparatively little attention has been given to their mode of action on the mycobacteria. The dearth of information is due in part to the evolution of more satisfactory drugs for tuberculosis, and in part to the fact that *Mycobacterium leprae* has not been cultivated *in vitro*. Although the usual techniques for the investigation of drug action cannot be applied to the leprosy bacillus, at least two means are available for gaining insight into the probable effects of therapeutic compounds in this disease. 1. While there may be differences in the mechanisms whereby a compound may exert bacteriostatic or bactericidal action on unrelated classes of microorganisms, the different species of the mycobacteria share many fundamental processes. Studies on the action of antileprosy drugs on cultivable mycobacteria, therefore, may afford clues concerning the alterations induced in *M. leprae*. 2. Meanwhile, methods are being developed for comparing the properties of the leprosy bacillus in untreated and treated patients, without necessity of cultivating the bacillus *in vitro*.

This paper describes the *in vitro* effect of DDS on the oxidative metabolism and the reproduction of certain readily-propagated mycobacteria. Although it has been suggested that some of the substituted derivatives of this compound are effective in leprosy without breaking down to the parent sulfone ([5]), an understanding of the action of 4:4'-diaminodiphenyl sulfone seems a prerequisite to further study.

In order to ascertain whether the action of DDS might interfere primarily with energy transformations or, on the contrary, with other processes essential to growth, advantage was taken of certain properties of mycobacteria which grow rapidly. A saprophyte (*M. phlei*) and a pathogen for frogs (*M. ranae*) were chosen for the following reasons.

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1. Their ability to oxidize rapidly a broad variety of substrates could permit sensitive indication of pathways which might be impeded by DDS. 2. Their versatile use of simple substrates for growth could be blocked only by interference with a fundamental process. 3. The high frequency of mutants exhibiting resistance to isonicotinic hydrazide and streptomycin offered maximal likelihood of obtaining DDS-resistant mutants which might differ significantly from the parent organisms.

The evidence accumulated thus far indicates that the action of DDS is not expressed (as in the case of isonicotinic hydrazide and streptomycin) by an interference with energy-yielding processes. A point of particular interest is that the growth-limiting action of DDS, although reversible and relatively inefficient, interferes with synthesis in a manner which is not circumvented by exceptional capacities to produce mutants.

MATERIALS AND METHODS

Mycobacteria.—The HMS strain of *M. phlei,* and the Trudeau strain of *M. ranae*: stock cultures on agar slants.

Basal media.—Basal media for growth curves and suspended inocula contained (grams/liter): K_2HPO_4 2, $MgSO_4$ 0.015, $FeSO_4$ 0.0015, Difco purified casamino acids 15, glucose 9, fumaric acid 1 (neutralized with ammonia), phenol red 0.02; also 3 cc. per liter of a 10 per cent solution of Tween 80. The final pH was adjusted to 7.0. To this base, 1.5 per cent agar-agar was added to prepare plating media. In studies on pH the phosphate used above was replaced by a Na_2HPO_4 —KH_2PO_4 mixture at a final molar concentration of M/30.

DDS solutions and solvents.—For respirometry experiments, DDS solutions containing 1.5 μmol/cc. (372 μg/cc.) were prepared by dissolving the pure recrystallized sulfone (37.2 mgm.) in warm ethyl alcohol (1.45 cc.) and diluting carefully to 100 cc. with distilled water. Other concentrations were prepared in a similar manner. Solutions were also prepared using dioxane (1% by volume) instead of alcohol, but they were found less suitable because of the inhibitory effect of dioxane on respiration.

Respirometry.—Oxygen uptake was studied at 37°C by conventional methods ([22]) in a sensitive Warburg respirometer ([10]).

Studies on growth inhibition.—Inocula for agar plates were measured and spread with a platinum loop (0.02 cc.). Total growth in liquid media was determined by optical-density measurements. Numbers of viable organisms were estimated by platings in rolled agar tubes after suitable dilution (10^4-10^6).

DDS-resistant mutants.—These were sought by (*a*) heavy platings of *M. phlei* and *M. ranae* on agar, with subsequent transfers to plates containing equal or greater amounts of DDS, and by (*b*) prolonged exposure in liquid media containing appropriate levels of DDS. Details will be found under Results.

EXPERIMENTAL RESULTS

Effect of DDS on the oxidation of substrates.—In studies with *M. phlei,* alcohol 1.45 per cent/volume (solvent for DDS) enhanced respiration by approximately 20 per cent. This slight enhancement was largely obliterated when 50 or 100 μg/cc. of DDS was added.

Succinic, acetic and stearic acids (in the presence of alcohol) enhanced respiration 2-fold to 3-fold. The addition of 50 μg/cc. of DDS

reduced these rates of oxygen consumption by absolute values which approximated the inhibitory effect on oxidation of the alcohol which accompanied the DDS.

Similar results were observed in the presence of glucose, glycerol, lactate, pyruvate, glycine, glutamate, alanine, aspartate, cadaverine, human serum, and the liquid basal medium. These modest inhibitions were not increased by preincubating *M. phlei* in 50 μg/cc. of DDS, then rewashing in order to test *M. phlei* plus adsorbed DDS in the absence of alcohol but in the presence of selected substrates alone.

To test more adequately the possibility of a specific inhibition of alcohol oxidation, both *M. phlei* and *M. ranae* were harvested from a synthetic medium containing 1.5 per cent/volume of that substrate (alcohol) and the ammonium ion as a source of nitrogen. The growth of *M.phlei* required additional sources of carbon, and resulted in but slight expansion of the sluggish systems for oxidizing alcohol. *M. ranae*, on the contrary, grew with alcohol as the sole source of carbon, and yielded suspensions which oxidized alcohol at 9 times the endogenous rates. This occurred whether the alcohol was available solely as vapors of dilute solutions (0.25%/vol.) in the side-arms of Warburg vessels, or was dumped into the main reaction vessel. The presence of DDS at 93 μg/cc. caused but 5 to 10 per cent inhibitions of these oxidation rates.

In view of the foregoing observations, it was concluded that, although DDS exerted a consistently depressant effect, no circumstance had been found in which it interfered significantly with substrate oxidations.

Effect of DDS on mycobacterial growth.—The growth of heavy inocula of *M. phlei* and *M. ranae* on agar containing DDS confirmed the inability of this drug to produce clear-cut growth inhibitory end-points. While 0.3 μg/cc. was definitely inhibitory after 48 hours of incubation, some growth was always evident. From 1-4 μg/cc. were required to maintain this degree of restraint for 120 hours. Furthermore, slight growth occurred even at drug concentrations of 60 μg/cc.

Analysis of the influence of individual components in the agar medium demonstrated that no single component competed with DDS, and that failures to restrain growth were associated with completeness and efficacy of the media employed. As will be seen later, the failures to obtain stable end-points on agar containing DDS cannot be explained by inactivation of DDS, or by the emergence of DDS-resistant mutants.

Quantitative experiments on growth in the liquid medium containing DDS at 5 μg/cc.[4] proved more instructive (Fig. 1). Such experiments showed that DDS decreased the rates of logarithmic growth slightly but consistently, and that growth terminated only 1 or 2 logs below

[4] This concentration was adopted after surveying the drug levels employed by others (4,8,14,19,20).

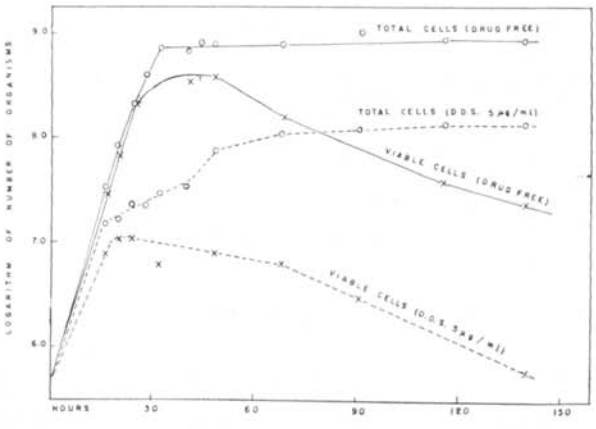

FIG. 1. Effect of DDS on the growth and viability of *M. phlei*. The peculiarities in the optical densities in the second curve (total cells, DDS 5 µg./ml.) were not due to DDS, and were often seen in control cultures. They are due partly to a phase in the growth cycle when there is maximal sensitivity to the influence of temperature and osmotic pressure (1). The difficulty later was minimized by making all observations at 37°C.

that attained in the absence of the drug. Since other experiments demonstrated that propagation of *M. phlei* in DDS medium did not remove any appreciable amount of drug from the medium, the normal decline in viability in the presence of DDS indicates its total lack of bactericidal action.

A definite effect of pH on DDS activity was demonstrated by buffering the liquid medium with M/30 PO_4 to ensure strict control of H ion concentration (Fig. 2). The growth of *M. phlei* in medium lacking DDS was not affected by pH values ranging from 6.0 to 7.5. Terminal optical densities after 90 hours in the presence of the sulfone at pH 7.5, however, were 0.6 logs below those observed at pH 6.0.

Further evidence of a consistent lag in the production of the sulfone effect was obtained by adding DDS to successive flasks at progressive stages during the growth of *M. phlei*. These experiments demonstrated that the onset of growth limitation was not determined by any particular phase in the normal growth cycle; that growth always proceeded for some generations without alteration; and that final crops were dependent upon the population attained prior to addition of the drug.

Growth for 24 hours in the presence of DDS did not alter the potentiality for growth in the absence of the drug. When cells were washed after this exposure, then incubated in fresh medium or in aliquots of used (filtered) medium lacking DDS, they promptly resumed growth at normal rates and produced crops proportional to the nutrients supplied.

The observation that large inocula of *M. phlei* appeared to delay and to minimize the effect of DDS was confirmed by inoculating fresh liquid medium with 1×10^5, 1×10^7, and 1×10^8 cells per cubic centimeter. All cultures, with or with out DDS, exhibited normal rates of growth during the first 24 hours. After 60 hours in the presence of DDS, growth of the small inoculum had terminated 1.5 logs below that in the control cultures. With 1×10^7 bacilli per cubic centimeter as the

inoculum, the DDS flasks were only 0.5 logs below the controls, while with 1×10^8 bacilli identical crops were produced. Similar, though less striking, effects were produced by incorporating large numbers of killed microorganisms in the standard viable inoculum.

Reversal of DDS action by PABA.—Prevention of the action of DDS by means of *p*-aminobenzoic acid (PABA) was readily demonstrated when *M. phlei* was grown in the presence of 10 μg/cc. DDS and varying concentrations of PABA. As shown in Fig. 3, equal weights of PABA nullified the inhibitory action of DDS, while 1 part of PABA (0.001 μg) to 10,000 parts of DDS produced a measurable decrease in the activity of the latter compound. The major gap between two families of curves occurred between PABA 0.01 and 0.001 μg/cc.

Failure to obtain DDS-resistant mutants.—Although the universal appearance of slight growths of *M. phlei* and *M. ranae* on DDS-agar

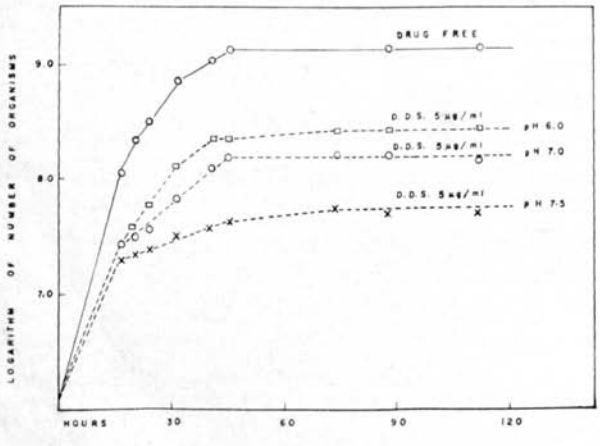

FIG. 2. Effect of pH on DDS inhibition of *M. phlei*. The same curve was obtained for the drug-free medium at pH 6.0, 7.0 and 7.5.

plates seeded with heavy inocula (possibly 1×10^8 bacilli) suggested mutational propensities of those microorganisms, the uniformity of such growths was not consistent with the small numbers of isolated mutant colonies which might be expected. Furthermore, the transfer of inocula from such growths to new agar containing equivalent or greater amounts of DDS led to restricted growth only and not to vigorous proliferation, as would have been expected with resistant colonies.

Much larger numbers of *M. phlei* were exposed to DDS in series of flasks containing 25 cc. or 75 cc. of liquid medium and inoculated with approximately 10^7 organisms per flask. As noted earlier, several logs of growth occurred in these cultures. In one series of flasks the drug concentration was progressively raised from 2 through 6 to 18 μg/cc., and in another it was maintained throughout at 5 μg/cc. In neither case was there any evidence of the development of resistant mutants, either in the liquid medium or after plating out separated organisms on agar

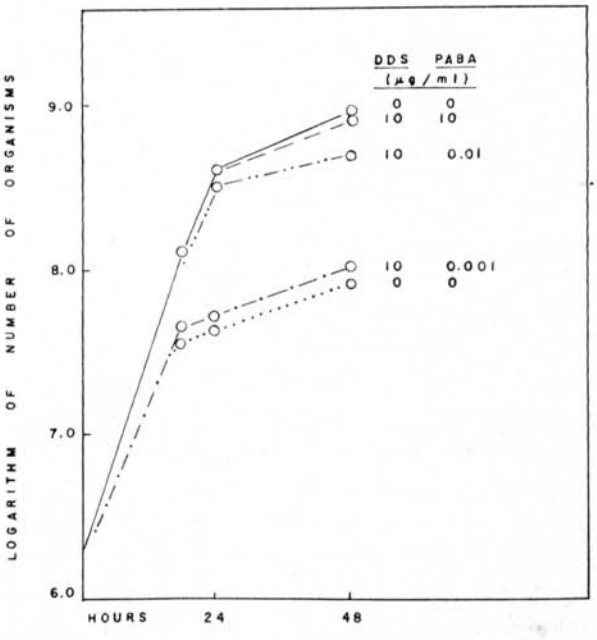

FIG. 3. Reversal of DDS inhibition of *M. phlei* by *p*-aminobenzoic acid (almost complete at a PABA:DDS ratio of 1:1 and still just detectable at a ratio of 1:10,000).

Note: The figures opposite the bottom curve should be "DDS 10, PABA 0" and not "DDS 0, PABA 0" as given.

containing DDS. Some of the flasks containing 18 μg/cc. DDS were kept under observation for 6 months.

It is estimated that, during the course of the work described in these and other experiments, over 10^{11} viable cells of *M. phlei* were exposed to varying concentrations of DDS for periods of from 5 days to 6 months, without obtaining a single colony which showed greater than average tolerance to this drug. Similar results were obtained with *M. ranae*.

DISCUSSION

Although the present study does not permit an explanation of the mode of action of DDS, it clarifies a few points concerning its influence on the metabolism and growth of mycobacteria. In the first place, it is evident that its effect is exerted in a manner which differs radically from that of compounds such as isonicotinic hydrazide or streptomycin. It fails to impede significantly the oxidation of substrate; it permits essentially normal rates of growth during a series of bacterial generations ([15]); and it is not bactericidal ([16, 17]). Its capacity to impose a limitation on full growth, therefore, is the major effect which has been demonstrated, but just how that limitation is effected is not evident.

The curves depicting growth in liquid media with and without DDS (Fig. 1) are of a type which would be produced if a vitamin-requiring microorganism were employed as the inoculum for media lacking and containing (respectively) the essential factor. The failure to observe clear-cut and stable end-points on DDS agar, the lag prior to limitation

of growth in liquid media, the immediate loss of restraint upon replacement of DDS-containing media with normal media, and the competitive effects of inoculum size, dead organisms or *p*-aminobenzoic acid, are consistent with the view that growth terminates only when the deficit of some metabolite essential for growth has become critical.

The competitive effect of *p*-aminobenzoic acid illustrates a resemblance between the action of DDS and the sulfonamides ([16]). This competition with DDS has been noted in the case of streptococci ([2, 12]), *E. coli* ([11]) and tubercle bacilli ([3, 6, 9, 21, 25]). Two groups of workers ([18, 23]) failed to detect this antagonism. While the production of PABA by *M. phlei* may explain many of the interrelationships here observed, it does not follow that competition with PABA is the only, or *in vivo* the most important, mode of action of DDS.

The greater effectiveness of DDS at slightly alkaline than at slightly acid pH values was noted earlier by Middlebrook and Lloyd ([13]), who suggested that the molecule may have greater penetrative power when the charge on the amine group has been partially neutralized.

Perhaps the single observation which has a bearing upon one of the unusual properties of sulfones during the treatment of leprosy is the failure to obtain resistant strains following exposure to the drug. Feldman and Hinshaw ([7]) found no evidence of sulfone resistance in tubercle bacilli recovered from guinea-pigs which had been treated up to 365 days. According to Wolcott and Ross ([24]), clinical relapse in leprosy patients treated almost constantly with sulfones seems to have occurred only after seven or more years of therapy. In the present study, no resistant derivates of *M. phlei* or *M. ranae* were obtained. In the case of each strain it was known that plating an inoculum in the order of 1×10^7 bacilli could be expected to yield colonies exhibiting significant resistance to isonicotinic hydrazide or to streptomycin. Although many multiples of such inocula were exposed to threshold concentrations of DDS, and incubations in some instances were extended to six months, the restraint imposed by DDS was of such character that no mutant forms were able to grow. The observations of Rist and associates ([16]) that reversal by PABA fails in higher concentrations of DDS indicates a second site of action. It may be this more absolute effect which makes it impossible to select mutants by the methods used.

SUMMARY AND CONCLUSIONS

1. The effect of 4:4′-diaminodiphenyl sulfone (DDS) on the metabolism and growth of *M. phlei* and *M. ranae* has been investigated.

2. The modest depression of oxidation of substrates, and of early phases of growth in a complete medium, indicate that interference with energy transformations is not a primary mode of action.

3. The absence of bactericidal action; the failure to block DDS-treated organisms transferred to fresh media; the inability to restrain growth during many bacillary generations; and the reversal of the DDS effect by large inocula, dead microorganisms or *p*-aminobenzoic acid, lead to the conclusion that DDS is not inhibitory in the ordinary sense of that word. It operates instead by limiting the synthesis or use of a metabolite which is essential for growth. This limitation is maximal at pH values above 7, and with small initial inocula.

4. The growth-limiting effect of DDS is in one respect more fundamental than that of compounds which are more inhibitory or bactericidal. It is exerted in a manner which circumvents the selective growth of drug-resistant mutants from the strains of mycobacteria used, which are prone to exhibit such mutations.

RESUMEN Y CONCLUSIONES

1. Se ha investigado el efecto de la 4:4′-diaminodifenil-sulfona (DDS) sobre el metabolismo y la proliferación del *M. phlei* y del *M. ranae*.

2. La modesta depresión de la oxidación de los subestratos, y de las fases incipientes de la proliferación en un medio completo, indica que la obstaculización de las transformaciones de la energía no constituye una modalidad primaria de la acción.

3. La falta de acción bactericida; la inobstrucción de los microbios tratados con DDS al ser trasladados a nuevos medios; la incapacidad para restringir la proliferación durante muchas generaciones bacilares; y la inversión del efecto de la DDS por grandes inóculos, microbios muertos o ácido *p*-aminobenzoico, llevan a la conclusión de que la DDS no es inhibidora en el sentido corriente de ese vocablo. Funciona más bien limitando la síntesis o utilización de un metabolito que es indispensable para el crecimiento. Esta limitación alcanza su máximo con cifras del pH superiores a 7, y con pequeños inóculos iniciales.

4. El efecto inhibidor del crecimiento debido a la DDS es en un sentido más fundamental que el de los compuestos que son más inhibidores o bactericidas. Se produce de un modo que elimina la proliferación selectiva de mutantes fármacorresistentes de las cepas de micobacterias usadas, las cuales son excepcionalmente versátiles en ese respecto.

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