A STUDY OF THE ACTION OF SULFONES ON THE METABOLISM OF MYCOBACTERIA[1]

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Although 4:4′-diaminodiphenyl sulfone (DDS) and its derivatives have long been used for the treatment of leprosy, comparatively little attention has been devoted to the fundamental study of their mode of action on the mycobacteria. The absence of laboratory cultures of *M. leprae* prevents the application of the usual techniques on that microorganism, and the research worker is left with two alternatives: (1) to study the action of the drug on related mycobacteria, or (2) to develop methods which can be applied to the relatively small numbers of bacilli that can be obtained from leprosy lesions in human patients. The present paper is an attempt to summarize some results obtained with cultured mycobacteria, and to indicate methods being developed to obtain direct results with *M. leprae.* The detailed results are being published jointly with John H. Hanks and Clarke T. Gray.

The study with cultured organisms has been conducted with a saprophyte, *M. phlei,* and a pathogen for frogs, *M. ranae,* and has been limited to the parent sulfone, DDS. An attempt has been made to measure the effect of the drug on energy transformations in the bacteria as measured by oxygen consumption, and on synthetic processes as measured by bacterial growth.

Effect of DDS on the oxidation of substrates.—In studies using a Warburg respirometer the low solubility of DDS in water made it necessary to add a water-miscible organic solvent, and for most purposes ethyl alcohol (1.45% by volume) was found satisfactory. Alcohol is itself slowly metabolized by *M. phlei,* and its oxidation was found to be markedly inhibited by DDS. Although succinic, acetic and stearic acids enhanced respiration two- to three-fold, the addition of DDS in a concentration of 50 μgm./cc. reduced these rates of oxygen consumption by less than 10 per cent, and the absolute value of the inhibitory effect was in no case greater than that obtained with alcohol itself (Graph 1).[2] This suggested that there might be some specific inhibition of alcohol metabolism—a view which, however, was not supported by further experiments. The respiration studies were continued with a wide range of substrates, but in no case was marked inhibition by DDS demonstrable. It appears that DDS exerts such a

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[2]The graphs referred to were not included with the manuscript obtained, but will appear in the paper as published in the transactions of the Congress.

slight (nonspecific) inhibitory effect on the oxidative metabolism of the mycobacteria studied that its efficiency as a drug could hardly be explained in these terms.

Effect of DDS on the growth of mycobacteria.—Although an effect of DDS on the growth of *M. phlei* and *M. ranae* on agar plates could be demonstrated, the most instructive results were obtained with growths in a synthetic liquid medium. Measurements of total growth (live and dead cells) were made by optical density methods, and of viable organisms at any stage by sampling techniques. Two facts emerge from the results of a growth experiment using a sulfone concentration of 5 μgm./cc. (Graph 2): (1) the DDS inhibition is not very apparent in the early stages of growth, and even finally is less than one log (i.e., 10X), (2) the effect of DDS is largely bacteriostatic, not bacteriocidal, as evidenced by the parallelism of the two curves for numbers of viable organisms.

By strongly buffering with phosphate the medium used for growth, it was possible to study the effect of pH on sulfone activity. The rate of growth of *M. phlei* is not appreciably affected by pH changes within the range 6.0 to 7.5, whereas it was shown that the sulfone inhibition was doubled by a change from pH 6.0 to 7.5. Intermediate values were obtained for pH 6.5 and 7.0 (Graph 3). The charge on the amine group, and to a lesser extent on the sulfone group, will be influenced by the pH, and consequent variation in chemical reactivity is to be expected. Middlebrook and Lloyd, in a study of the effect of DDS during *in vitro* experiments in tuberculosis, noted a similar phenomenon and suggested that the molecule might have a greater penetrating power with the amine group in the uncharged state.

The observation that large inocula of mycobacteria appeared to delay or minimize the effect of DDS was confirmed in a study of the growth of *M. phlei* from initial cell concentrations ranging from 10^5 to 10^8 cells per cubic centimeter. Over this range the inhibiting effect of DDS decreased from a factor of 15X to zero (Graph 4). Even the addition of dead bacteria had some effect in nullifying sulfone activity, although to a much less marked extent. This effect suggested the possibility of *p*-aminobenzoic acid antagonism, which is well known with the sulfonamides and has been reported by some workers with DDS. *M. phlei* were therefore grown in liquid medium containing 10 μgm./cc. of DDS and varying concentrations of *p*-aminobenzoic acid. The results (Graph 5) make it clear that such an antagonism does exist, and that as little as 1/1000 of the concentration of PAB (with respect to DDS) will reduce the inhibiting action of the sulfone by more than one-half. The production of PAB by the bacteria offers an explanation of the incompleteness of sulfone inhibition.

Failure to obtain sulfone-resistant mutants.—If PAB antagonism were the only mode of action of the sulfones, resistant mutants might be expected to emerge with comparative frequency. A further indication of

the possibility of producing sulfone-resistant strains was the observation that some growth of mycobacteria continued even in the presence of high concentrations of DDS, and experiments were therefore devised to isolate if possible sulfone-resistant organisms. In one experiment *M. phlei* was grown for 48 hours in a liquid medium containing 2 μgm./cc. of DDS, and then for a further 22 hours with 6 μgm./cc. of DDS. Specimen portions of the resultant bacteria were spread on agar plates containing DDS concentrations of zero, 5 and 50 μgm./cc. Provided small inocula were used, growth was very slight on the sulfone-containing plates. Moreover, bacteria grown in the drug-free liquid medium showed more growth on the DDS plates than did those grown in sulfone-containing medium. This is the reverse of what would be expected if DDS-resistant strains arose, and suggests that DDS is capable of doing some general, if slight, damage to reproductive systems in the bacteria. Further portions of the bacteria were allowed to grow for long periods in medium containing 18 μgm./cc. of DDS, but in no case was a resistant mutant detected. In this experiment a total of about 5×10^{11} cells were exposed to the drug.

In another experiment *M. phlei* was grown in liquid medium containing 5 μgm./cc. of DDS until the population had reached about 3×10^{11} cells. Suitably diluted samples were spread on agar plates containing 1 μgm./cc. of DDS, but in no case were sulfone-resistant colonies detected. Similar results were obtained with *M. ranae.*

When growth of *M. phlei* in the presence of sulfone was continued for 7 days, considerable changes in morphology were noted. In particular there were a loss of acid-fastness and a tendency to grow long forms, suggesting a hindrance of cell division.

Experiments with M. leprae.—It has been shown by Hanks that measurement of dehydrogenase activity of *M. leprae murium* provides a reliable index of the ability of the bacteria to infect fresh animals. Dehydrogenase activity is readily measured by tetrazolium salts, and in the case of cultured mycobacteria the colored formazan produced is deposited in the actual bacterial bodies, giving the appearance of a stained organism. In this way living and dead bacteria may readily be distinguished. Unfortunately, experiments with *M. leprae* gave largely negative results. It has been suggested by Hanks that this is due to a failure of the tetrazolium molecule to penetrate the lipid layer of the bacteria. However, there is some indication that under suitable conditions dehydrogenase may diffuse from the bacteria and react with tetrazolium salts in the surrounding medium.

In the present experiments a study of differing methods of separating the bacteria from tissue elements showed that no advantage was to be gained by elaborate techniques. The gain in purity of the specimen of *M. leprae* was offset by the loss of enzyme activity during manipulation. Moreover, experiments with healthy tissue indicated that interference by tis-

sue dehydrogenase was only slight. Normally, a small piece of a nodule from a lepromatous patient was macerated by cutting in isotonic phosphate solution at pH 7.4. The supernatant bacterial suspension was incubated at 37° with 0.2 per cent solutions of tetrazolium blue or violet under anerobic conditions for periods of 24-96 hours; it was found advantageous to add penicillin (10,000 units per cc.) to restrain growth of contaminants. In a number of experiments the development of color in specimens from untreated lepromatous cases was obviously greater than that in similar samples from patients receiving sulfone therapy, but a quantitative method of assessing this difference has only recently been developed. In this method the solid matter is separated by centrifugation, the colored formazan extracted with acetone, and the color densities compared photoelectrically. The results obtained to date with tetrazolium blue are summarized in Table 1. However, data of many more specimens are required before any assessment can be made of the significance of these quantitative results.

TABLE 1.—*Color production by leproma preparations from tetrazolium blue.*

Treatment of case (DDS)	Photoelectrometer readings[a]		
	Experiment 1	Experiment 2	Experiment 3
None	35	44	45
		44	30
			29
6-10 weeks	28	30	
6-12 months	22	21	
	23	18	
		15	
4-8 years		14	
		13	
10 years	9		

a Each figure represents a specimen from a different case. The activity is expressed in arbitrary units of color production.

SUMMARY

1. Results of the study with cultured mycobacteria suggest that:

(*a*) DDS exerts little effect on energy transformations in the mycobacteria.

(*b*) The action of the drug is bacteriostatic and not bacteriocidal.

(*c*) Although the effect of DDS can be reversed by *p*-aminobenzoic acid, this is unlikely to be the sole mode of action in view of the lack of evidence for the development of sulfone-resistant mutants.

2. Using tetrazolium salts it is possible to detect changes in the metabolic activity of *M. leprae* following treatment with DDS, but further experiments are required to establish this on a quantitative basis.

RESUMEN

1. Sugieren los resultados de este estudio con micobacterias cultivadas que:

(*a*) La DDS ejerce poco efecto sobre las transformaciones de la energía en las micobacterias.

(*b*) La acción de la droga es bacteriostática y no bactericida.

(*c*) Aunque cabe invertir el efecto de la DDS con el ácido *p*-aminobenzoico, es improbable que esto sea la única forma de acción, en vista de la falta de signos de que haya mutantes sulfonorresistentes.

2. Usando las sales de tetrazolio es posible descubrir las alteraciones de la actividad metabólica del *M. leprae* durante el tratamiento con la DDS, pero se necesitan más experimentos para establecer esto a base cuantitativa.