THE NATURE OF ANTITUBERCULOUS ACTION OF SULFISOXAZOLE. REPORT I

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(Received September 17, 1957)

It is well known that sulfathiazole represents a marked *in vitro* tuberculostatic action (DOMAGK)[1]. Recently, TSUKAMURA reported that 3,4-dimethyl-5-sulfanilamide-isoxazole (sulfisoxazole) has a tuberculostatic activity similar to sulfathiazole and the drug prevents the *in vitro* emergence of streptomycin resistance and isoniazid resistance as well as sulfathiazole[2,3]. NAITO[4], SAITO, *et al.*[5], and USHIBA, *et al*[6]. utilized the combination of isoniazid and sulfisoxazole for treatment of pulmonary tuberculosis and reported that isoniazid-sulfisoxazole therapy gave a marked clinical effect on tuberculous patients. Therefore, it appears desirable to make clear the nature of tuberculostatic action of sulfisoxazole. The present paper concerns with this problem.

Materials and Methods

Mycobacterium tuberculosis var. *hominis*, strain H37Rv, was used through the present study. The medium used was TATSUGI-OGAWA's egg medium. The composition of the medium is as follows: Basal solution (1% KH_2PO_4 and 1% sodium glutamate), 100 ml; Whole eggs, 200 ml; Glycerol, 6 ml; 2% aqueous solution of malachite green, 6 ml. The medium was poured in 8 ml quantities into each tube, 18×170 mm. The medium was slanted by sterilization at 88°C for 60 minutes. The length of slants was 11~12 cm.

Sulfisoxazole (3,4-dimethyl-5-sulfanilamide-isoxazole) used in the study was the one manufactured by Shionogi Co. (Sulfazin). The drug was dissolved by adding with 1% NaOH-solution and added to medium prior to sterilization.

Six week cultures grown as discrete colonies of the test strain was used for preparing cell suspensions. The culture was shaken with glassbeads for 10 minutes and suspended with saline. Ten-fold dilutions were made of the cell suspension and 10^0 (the original suspension)-to 10^{-6}-dilutions were prepared. Appropriate dilutions were utilized for inoculation.

Inoculation was made with a large whirled loop delivering 0.02 ml onto medium slants, for the method of inoculating cell suspensions with the whirled loop had been found by us to give the smallest variation of colony numbers[7].

Generation time was calculated according to YOUMANS and YOUMANS[8]. The generation time was found by substituting the growth rate constant, K, into the formula: $G=\log 2/K$.

To estimate the growth rate constant, five sets of tubes were inoculated with each of the 10^{-1}-to 10^{-5}-dilutions and one set consisted of five tubes. The inoculated tubes were incubated at 37°C and examined daily at approximately the same time, and a record was kept when growth was first noted.

Results

1) The effect of sulfisoxazole on the growth rate of *Mycobacterium tuberculosis*.

The generation time was measured on various concentrations of sulfisoxazole, and the results are shown in Table 1 and Figure 1. As shown in the figure, a linear relationship has been found between the generation time and the sulfisoxazole concentration, and the generation time has been found to be a function of the sulfisoxazole concentration. The results indicate that the nature of action of sulfisoxazole on *M. tuberculosis* is to delay the generation time. It is very probable that the delayed generation time results from the inhibition of synthesis of new cell material as purine derivatives, *etc.*

Table 1. Generation time of *Mycobacterium tuberculosis* var. *hominis*, H 37 Rv, on various sulfisoxazole concentrations.

Medium	Growth rate constant	Generation time
Sulfisoxazole 0 mcg/ml	0.852	8.48 hours
Sulfisoxazole 1 mcg/ml	0.755	9.55
Sulfisoxazole 5 mcg/ml	0.445	16.2
Sulfisoxazole 10 mcg/ml	0.309	23.3

Table 2. Time of first appearance of visible colonies on tubes giving discrete colonies. (Test strain: *M. tuberculosis* var. *hominis*, H 37 Rv)

Sulfisoxazole concentration	Time in days of first appearance of growth		
	Mean	Standard deviation	No. of replicates
0 mcg/ml	13.0	0.00	5
1	14.4	0.55	5
5	21.4	0.55	5
10	27.0	0.00	5

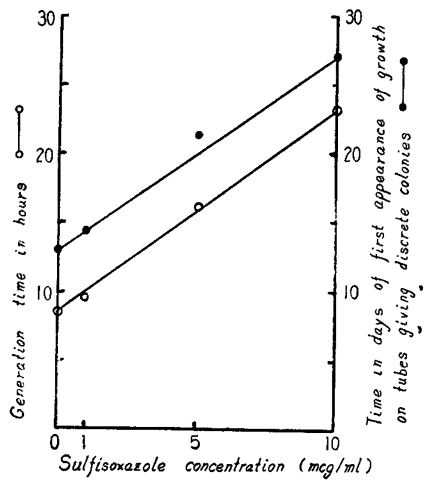
Table 3. Number of survivors on various sulfisoxazole concentrations. (Test strain : *M. tuberculosis* var. *hominis*, H37Rv. Number of survivors (colonies) were calculated after 4 week incubation.)

Sulfisoxazole concentration	Mean (*1)	SD (*2)	No. of repl. (*3)	95% confidence limits (*4)
0 mcg/ml	54.5	10.2	5	47.26—61.74
1	42.1	11.7	5	33.78—50.42
5 (*5)	35.5	13.5	5	32.80—51.70
10 (*6)	23.8	5.14	5	20.14—27.46
20	0.0	—	5	—

(*1) Mean of colonies
(*2) Standard deviation
(*3) Number of replicates (tubes)
(*4) 95% confidence limits derived from (mean) ± "t" x (standard error)
(*5) Small colonies
(*6) Minute colonies.

Time of first appearance of visible colonies were also recorded on tubes giving later discrete colonies when the tubes were incubated until the end of the forth week of incubation, since this time has been considered to be an index of the growth rate. Of course, it is necessary to observe the first appearance of growth on tubes inoculated with the same inocula. This was made by observing it on tubes giving later discrete colonies. The tubes had been inoculated with the same 10^{-5}-dilution of the cell suspension. The results obtained are shown in Table 2 and Figure 1, and the numbers of colonies on the tubes used for determining the time are shown in Table 3. As shown in Figure 1, a linear relationship has been found between the time of first appearance of growth on the tubes and the sulfisoxazole concentration, and the curve was quite parallel with the curve of the generation time.

FIG. I



2) The antagonistic effect of para-aminobenzoic acid on sulfisoxazole and sulfathiazole.

HATTA, *et al.*[9] reported that the antagonistic ratio of *p*-amino-benzoic acid (PABA) against sulfisoxazole was greater than that against sulfathiazole in ***Salm. typhi murium*** and ***Aerobacter aerogenes***. Accodingly, it appeared important to observe the ratios in ***M. tuberculosis***.

Sulfisoxazole, sulfathiazole (Sulzol, Takeda Co.) and sodium paraaminosalicylate (PAS, Shionogi Co.) were added into media in various combinations with PABA. Inoculum permitting actual counts (ca. 150 viable cells per tube) were used to obtain accurate results. After 4 week incubation period, drug concentrations giving the same growth as in control tubes were recorded. The results obtained are shown in Table 4. As shown in the table, the antagonistic ratio was 1 : 1 in PAS, 1 : 10 in sulfisoxazole, and 1 : 50 in sulfathiazole under the conditions tested. The ratio was much larger in sulfisoxazole than in sulfathiazole. The results suggest that sulfisoxazole is better for clinical use than sulfathiazole.

(Appendix) The same results have been obtained in *Mycobacterium avium* (See Table 5). The anta-

Table 4. The antagonistic effect of *p*-aminobenzoic acid (PABA) on sulfisoxazole, sulfathiazole and PAS in *Mycobacterium tuberculosis* var. *hominis*, H37Rv.

PABA	Sulfisoxazole	Sulfathiazole	PAS
0	$M/5\times10^4$	$M/5\times10^4$	$M/5\times10^5$
$M/10^5$	$M/10^4$(1:10*)	$M/2\times10^3$(1:50*)	$M/10^5$(1:1*)
$M/10^4$	$M/10^3$(1:10*)	$M/2\times10^2$(1:50*)	$M/10^4$(1:1*)

The molar concentrations in the table indicate the ones permitting the same growth as in control containing no drug.
* Ratios of PABA against tuberculostatica.

Table 5. The antagonistic effect of *p*-aminobenzoic acid (PABA) on sulfisoxazole and sulfathiazole in *Mycobacterium avium*, strain Jucho.

PABA	Sulfisoxazole	Sulfathiazole
0	$M/15^5$	$M/10^5$
$M/10^7$	$M/10^5$ (1:100*)	$M/10^4$ (1:1,000*)
$M/10^6$	$M/5\times10^4$(1: 20*)	$M/2\times10^3$(1: 500*)
$M/10^5$	$M/5\times10^3$(1: 20*)	$M/5\times10^2$(1: 200*)

Test medium : Modified Sauton medium (Asparagin was substituted by sodium glutamate), pH 7.0, 2 ml in each tube.
Inoculum : approximately 10^5 viable cell units per tube.
The molar concentrations in the table indicate the ones permitting the same growth as in tube containing no drug.

gonistic ratio of PABA against sulfisoxazole was much greater than that against sulfathiazole in *M. avium*.

Discussion

The results obtained indicate that the generation time of the organism is a function of the sulfisoxazole concentration, and it is, therefore, expected that the colony size is also a function of the drug concentration and it becomes larger with the incubation period. This was true as shown in Table 3 and in the 2nd report of the study. It is also expected that growth becomes visible even on media containing higher concentrations of the drug, when a large inoculum is utilized, and the growth does not always consist of resistant variants but of sensitive cells. This was also found to be true by the 2nd report.

The nature of tuberculostatic action of sulfathiazole had been indicated by us previously to be in delaying the growth of *M. tuberculosis*[2,10,11]. It has been more accurately proved in the present paper that the nature of tuberculostatic action of sulfisoxazole also is of the same nature.

Summary

The nature of antituberculous action of 3,4-dimethyl-5-sulfanilamide-isoxazole (=sulfisoxazole) on *Mycobacterium tuberculosis* var. *hominis* has been studied. Sulfisoxazole delays the generation time of *M. tuberculosis*. The generation time is a function of the sulfisoxazole concentration and a linear relationship exists between the generation time and the drug concentration. The result suggests that sensitive cells can multiplicate and the growth of sensitive cells can be visible even in higher drug concentrations, when a large inoculum is utilized. The antagonistic ratio of *p*-aminobenzoic acid against sulfisoxazole is 5-times greater than that against sulfathiazole in *M. tuberculosis*. It has been, therefore, suggested that sulfisoxazole is a much better antituberculous compound than sulfathiazole.

The authors wish to express their appreciation to Dr. R. KATSUNUMU and Prof. HIBINO for their kind discussion.

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