tration of 0.066 M was chosen, and equimolar solutions of urea, thiourea, and guanidine (in the form of its sulfate) were made up in sterile SG medium. One ml of sulfathiazole solution (10^{-3} M) was added to 99 ml portions of the above solutions for the mixed drug solutions. These, together with SG medium (control) and 10^{-5} M sulfathiazole in SG medium, were added in 10 ml amounts to sterile tubes. One-half ml of a 1:2.5 dilution of an overnight culture of *E. coli* was added to each tube, giving an initial population of about 1.1 x 10^6 organisms per ml. The suspensions were incubated at 37°, and their densities determined at various times. The result of this experiment is shown in Fig. 2, each point representing an average of 4 or 5 determinations.

It can be seen from Fig. 2 that, over the entire period of 24 hours, the order of increasing synergistic action is urea, thiourea, and guanidine. At 24 hours, the time chosen by Tsuchiya *et al.*,[2] the picture is more favorable for the latter two compounds. The urea, in the presence of the greater number of organisms (which is essentially a dilution from the standpoint of a single enzyme molecule) shows a growth stimulating effect. The presence of thiourea (0.066 M) brings about a 22%, and guanidine a 34% growth decrease, in addition to that caused by the sulfathiazole (10^{-5} M), which was about 13%. The figures on thiourea and guanidine are on the basis of the growth of the organism in the presence of sulfathiazole (10^{-5} M), which is the related control.

Summary. Guanidine and thiourea, in this order, have been found to be much more active than urea (all at the same molar concentration), when used jointly with sulfathiazole in inhibiting the growth of *E. coli* on a simple medium. The action is a synergistic one.

The synergistic action of urea with sodium sulfadiazine on *E. coli* has been confirmed, using the conditions defined by Strakosch and associates.

[4] Kohn, H. I., and Harris, J. S., *J. Pharm. and Exp. Therap.*, 1943, **77**, 1.

14324

Synergism of Urea and Sulfathiazole on *E. coli*. Effect of Inoculum Size.

S. W. LEE, JEANNE A. EPSTEIN, AND E. J. FOLEY. (Introduced by Marion B. Sulzberger.)

From the Wallace Laboratories, Inc., New Brunswick, N.J.

In a previous paper (see companion article) the varying opinions of Tsuchiya *et al.*[1] and Kirby[2] concerning the nature of the action of urea with the sulfonamides upon *E. coli* has been mentioned. In conjunction with other problems, a few experiments have been carried out in this laboratory which are considered to bear on this problem.

It should first be pointed out that the experiments of Kirby[2] were not carried out exactly as were those of Tsuchiya *et al.*, and because of these variations the results should probably not have been contrasted. The obvious points of difference in the two series of experiments were media and inoculum size. The medium of Kirby contained sugar, salts and casamino acids, Difco, whereas that of Tsuchiya *et al.* consisted only of salts and glucose, similar in nature to that which has been used in experiments reported here (medium SG[3]).

The much greater departure in conditions, it is felt, concerns the inoculum size. Tsuchiya *et al.* worked with the large initial population of about 800,000 organisms per ml. Kirby

[1] Tsuchiya, H. M., Tenenberg, P. J., Strakosch, E. A., and Clark, W. G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 245. (See also previous papers.)

[2] Kirby, W. M. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 109.

[3] Kohn, H. I., and Harris, J. S., *J. Pharm. and Exp. Therap.*, 1943, **77**, 1.

TABLE I.
Sizes of Inocula of *E. coli* and the Bacteriostatic Action of Urea, Sulfathiazole and Their Combination.

	Optical densities									
Initial Population	$6x10^7$ org/ml		$6.5x10^6$ org/ml		$1.3x10^6$ org/ml		$6.5x10^5$ org/ml		$6.5x10^4$ org/ml	
Hrs after inoculation	5	21	5	21	5	21	5	21	5	21
Substances added to medium										
Control, no drug	.28	.32	.18	.36	.06	.20	.03	.19	0	.14
Sulfathiazole (STA) 10^{-5} M	.30	.35	.05	.23	.01	.22	—	.16	0	.01
Urea 0.2%	.28	.28	.17	.26	.05	.28	.02	.25	0	.24
" 0.5%	.34	.29	.16	.32	.05	.26	.02	.28	0	.26
" 1.0%	.29	.36	.11	.40	.02	.33	0	.33	0	.28
" 2.0%	.28	.35	.09	.24	.02	.28	0	.25	0	.26
(STA) 10^{-5} M + Urea .2%	.29	.34	.05	.28	0	.22	0	.15	0	0
" " " " " .5%	.30	.33	.05	.23	0	.22	0	.13	0	0
" " " " " 1.%	.27	.32	.05	—	0	.09	0	.02	0	0
" " " " " 2.%	.21	.26	.022	.12	0	.03	0	.02	0	0

does not mention the inoculum size used in his work, but the nature of his graphs indicates it to have been rather small.

The effect of the size of the inoculum on the results obtained in testing bacteriostatic substances is well known.[4] In general, small inocula are considered to give unsatisfactory or misleading results. The work on this phase of the problem of urea-sulfonamide synergism is given in this report.

Method. The methods used were essentially those previously reported.[5] Growth curves of *E. coli* were measured turbidimetrically at critical times, 5 and 21 hours, using a Coleman universal spectrophotometer with the wage-length dial set at 600 mμ. The optical densities are given as such, a value of 0.30 corresponding to a population of 6.5×10^8 organisms per ml. The population, as determined by plate count, is roughly linearly related to the density.

In the experiments, 10 ml of medium SG containing the desired concentration of the drugs was added to a series of sterile tubes. The varying inocula were added to these tubes. The culture (18-hour) used for inoculation contained 6.5×10^8 organisms per ml, and volumes of from 1 ml of this culture to 0.10 ml of its 1:100 dilution were added to the tubes, giving the initial population indicated in Table I. Each determination was done in triplicate. The tubes were incubated at 37°C.

Results. Table I indicates the marked effect of inoculum size on the results obtained. With very large inocula the growth stimulatory effect of urea alone is manifest (*cf.* Tsuchiya *et al.*), while with the small size it becomes bacteriostatic (*cf.* Kirby). The effect of sulfathiazole (10^{-5} M) is comparable. The combination of these two always has a synergistic effect—slight with large inocula, but very pronounced with the smaller. The 21-hour data from initial population of 1.3×10^6 organisms per ml corresponds well to that of Tsuchiya *et al.* Though the urea alone acts to retard growth during the first few hours, its action with sulfathiazole must still be considered synergistic.

It has been shown that part of the divergent opinions concerning the conjoint action of urea must result from the use of different experimental conditions.

Summary. When a salt-glucose medium is used, the size of the inoculum of *E. coli* has been shown to play the dominant part in studies concerning the effect on growth rates of urea, sulfathiazole, and their combinations. The action of these two alone has been shown to vary from stimulatory, with the very large inocula, to markedly bacteriostatic with the smaller. When used together, sulfathiazole and urea (in several concentrations) act synergisticly with all inocula sizes, the combined effect being much greater with the smaller inocula.

[4] MacLeod, C. M., and Mirick, G. S., *J. Bact.*, 1942, **44**, 277.

[5] Lee, S. W., and Foley, E. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 243.