SULPHANILAMIDES

Part II. *In vitro* Synergism with Anionic Surface-Active Compounds

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Combined administration of two or more drugs in the chemotherapy of severe infections has frequently shown synergistic effects, permitting the use of smaller doses of each compound and preventing or reducing toxic effects and reactions.[1–5] Successful treatment of mixed infections and prevention of acquired drug resistance by micro-organisms are also possible.[6–7] These observations have been made on sulphanilamides and antibiotics such as penicillin and streptomycin.[8–9]

Surface-active compounds of certain types inhibit bacterial metabolism in high dilutions,[10–14] and they have been used largely to increase the potency of germicidal and disinfecting agents.[15–18] There are a few recent reports concerning their synergism with chemotherapeutics.[19–21] The low solubilities of sulpha drugs, the high concentrations needed for bacteriostatic action in natural habitats in comparison with penicillin and similar antibiotics and, in particular, the rapid development of resistance by most micro-organisms make it desirable to use them in combination with other antibacterial agents. This study on the additive bacteriostatic effects *in vitro* of certain surface-active compounds and sulpha drugs was therefore undertaken as part of investigations in progress[22] on the synthesis of compounds possessing antibacterial action and surface activity.

The recognition that conventional peptone media contain sulphanilamide inhibitors obscuring the complete manifestation of potential drug activity[23–25] has led to the use of various synthetic media,[26–29] although the latter are obviously less suitable for bacteriostatic studies on the basis of which *in vivo* tests are to be planned. Both synthetic and peptone media have been employed in the present work.

Methods

Bacteriological tests were carried out against *Staphylococcus aureus* Rosenbach, *Escherichia coli* (Migula) Castellani and Chalmers, and *Eberthella typhosa* (Zopf) Weldin. The culture media used were nutrient broth (Stearn's peptone, 1 per cent.; beef extract Lab. Lemco, Oxo, 0·4 per cent.; and sodium chloride, 0·5 per cent.) and the synthetic medium[29] of Muir, *et al.*

($NH_4H_2PO_4$, 0·1 per cent.; K_2HPO_4, 0·1 per cent.; sodium citrate 0·2 per cent.; $MgSO_4$, 0·02 per cent.; and NaCl, 0·5 per cent.), both at pH 7·6. The media were tubed in 5 c.c. quantities and stock slants of the test organisms were maintained on 2 per cent. agar with the respective media through fortnightly transfers. Test cultures were transplanted into the media and incubated at 37°. The organisms grew well in the artificial medium only after a period of adaptation by repeated subculturings (*cf.* ref. 28).

Since young cultures, during their logarithmic phase of growth, are more susceptible to drug action than the cells from older cultures,[28, 30, 31] 16–18 hour cultures of the organisms were employed for the tests unless otherwise stated.

The inoculum for the tests consisted of cells grown in the medium to be used and washed twice by centrifugation in the same medium; water, saline, or buffer was not used for washing since they were likely to kill or injure some of the cells. The size of the inoculum was normally a 2 mm. loopful from a standard suspension which gave the same density in a Klett-Summerson photo-colorimeter and corresponded to 5,000–10,000 cells. A small variation, not allowed for, in the volume of medium carried by the standard loop is likely to have been caused by the alterations in surface tension brought about by the surface-active agents where present.

The drugs tested were sulphanilamide, sulphadiazine, sulphapyridine, sulphathiazole, sulphamerazine, and sulphaguanidine. The surface-active agents used were Igepon T (General Dyestuffs), sodium salt of N-oleyl N-methyltaurine; Aerosol OT (American Cyanamid), sodium dioctyl sulphosuccinate and Nekal BX (I.G. Farbenindustrie), sodium salt of a dibutylnaphthalenesulphonic acid. The drug or the surface-active compound to be tested was incorporated into the medium prior to tubing and, where necessary (as with certain of the sulpha drugs), a minimal amount of dilute hydrochloric acid was employed to facilitate solution of the drug, the pH being maintained at 7·6 ±0·2; there was no precipitation of the surface-active compound. Bacteriostasis was determined on the basis of the absence of visible growth at the end of 72 hours unless specified otherwise.

Effects of age of culture, size of inoculum and temperature.—Since time and temperature of incubation and number of bacteria inoculated, in addition to the type of medium, are important factors, which may influence the conclusions drawn from sulphanilamide studies *in vitro*,[31, 32] these were ascertained in the following experiments. Eighteen and 24-hour old cultures of *S. aureus* in peptone-broth and synthetic media were used as inocula in final dilutions of (*a*) 1 in 50, (*b*) 1 in 2,500, (*c*) 1 in 1,25,000 and (*d*) 1 in 6,250,000. A standard loopful of the undiluted culture in 5 c.c. of the medium corresponded approximately to a dilution of 1 in 250. Dilution (*d*) was

not employed with the synthetic medium. Observations for visible growth in presence of varying amounts of sulphanilamide were made at different time intervals after incubation at 37° and at 43°. The results are shown in Table I.

TABLE I

Effect of age of culture on the susceptibility of S. aureus *to sulphanilamide in relation to temperature and dilution*

Hours' incubation	Dilution of inoculum	Concentration of sulphanilamide (mg. per cenr)							
		Nil (control)		400		600		800	
Temp. of incubation		37°	43°	37°	43°	37°	43°	37°	43°
(i) Culture 24 hours : Peptone-broth medium									
16	a	+	−	+	±	−	−	−	−
	b	+	−	−	−	−	−	−	−
	c	−	−	−	−	−	−	−	−
	d	−	−	−	−	−	−	−	−
24	a	++	+	+	+	+	−	−	+
	b	++	+	±	−	+	−	−	+
	c	+	+	−	−	−	−	−	−
	d	+	+	−	−	−	−	−	−
48	a	++	++	++	++	+	+	+	−
	b	++	++	++	+	+	−	+	−
	c	+	+	+	−	+	−	±	−
	d	+	+	+	−	+	−	−	−
72	a	++	++	++	++	++	++	++	−
	b	++	++	++	+	++	+	+	−
	c	++	++	++	+	++	+	+	−
	d	++	++	+	+	++	−	+	−
(ii) Culture 18 hours : Peptone-broth medium									
16	a	+	−	−	−	−	−	−	−
	b	+	−	−	−	−	−	−	−
	c	−	−	−	−	−	−	−	−
	d	−	−	−	−	−	−	−	−
24	a	++	+	−	−	−	−	−	−
	b	++	+	−	−	−	−	−	−
	c	+	+	−	−	−	−	−	−
	d	+	+	−	−	−	−	−	−
48	a	++	++	+	±	+	+	+	−
	b	++	++	+	−	+	−	−	−
	c	++	++	−	−	−	−	−	−
	d	++	++	−	−	−	−	−	−
72	a	++	++	++	+	+	+	+	−
	b	++	++	±	+	+	+	+	−
	c	++	++	−	−	−	−	±	−
	d	++	++	−	−	−	−	−	−

TABLE I—(*Contd.*)

Hours' incubation	Dilution of inoculum	Concentration of sulphanilamide (mg. per cent.)							
		Nil (control)		400		600		800	
Temp. of incubation		37°	43°	37°	43°	37°	43°	37°	43°
(iii) Culture 24 hours : synthetic medium									
16	a	+	+	+	−	−	−		
	b	+	+	+	±	−	−		
	c	−	−	−	−	−	−		
24	a	+	+	+	+	−	−		
	b	+	+	±	+	−	−		
	c	+	+	−	−	−	−		
48	a	++	++	++	++	−	−		
	b	++	++	++	++	−	−		
	c	++	++	++	++	−	−		
72	a	++	++	++	++	+	−		
	b	++	++	++	++	−	−		
	c	++	++	++	++	−	−		
(iv) Culture 18 hours : synthetic medium									
16	a	+	+	+	±	−	−		
	b	+	+	+	−	−	−		
	c	+	+	−	+	−	−		
24	a	+	+	+	±	−	−		
	b	+	+	+	+	−	−		
	c	+	+	+	+	−	−		
48	a	++	++	++	++	−	−		
	b	++	++	++	++	−	−		
	c	++	+	++	++	−	−		
72	a	++	++	++	+	−	−		
	b	++	++	++	++	−	−		
	c	++	++	++	++	−	−		

− No growth. + Definite but small growth.

± Traces of growth. ++ Good to maximum growth.

With the peptone medium generally, the older the cells and the lower the temperature of incubation the less susceptible is the organism to the drug. The results at the higher temperature are relatively independent of the age of the culture and the concentration of the inoculum. With the synthetic medium, on the other hand, the effects of age of culture and size of inoculum are less pronounced at either temperature. The explanation

for this observation might be that, owing to the more steady and prolonged growth of the organism in the peptone-broth medium, the cells used in the different inocula vary somewhat in their stages of growth. Rose and Fox[33] consider that the bacterial cell contains a susbtance necessary for reproduction which is synthesized normally but not in presence of bacteriostatic concentrations of sulphanilamides. This would imply that with heavy inocula there is no bacteriostasis with the same effective concentration of drug as with more dilute inocula. Neter[34] has reported that the number of organisms in the inoculum does not affect the drug concentration needed for growth inhibition, provivded the studies are carried out at higher incubation temperatures.

In the following studies, the incubation temperature employed was normally 43° for the peptone-broth medium and 37° for the synthetic medium.

Minimum effective concentrations of sulpha drugs and surface-active compounds.—The results of antibacterial titre tests with the drugs and the wetting agents are given respectively in Tables II and III.

TABLE II

Minimum inhibitory concentrations of sulpha drugs

Sulpha drug	Organism	Synthetic medium		Peptone broth medium	
		37°	43°	37°	43°
		(mg. per cent)			
Sulphanilamide	*S. aureus* ..	600	600	900	800
	E. coli ..	500	600	600	600
	E. typhosa ..	500	500	750	800
Sulphadiazine	*S. aureus* ..	22	20	200	300
	E. coli ..	20	15	300	400
	E. typhosa ..	15	15	300	300
Sulphapyridine	*S. aureus* ..	20	18	300	300
	E. coli ..	22	20	500	500
	E. typhosa ..	18	15	400	400
Sulphathiazole	*S. aureus* ..	15	20	300	200
	E. coli ..	15	15	450	400
	E. typhosa ..	20	20	300	400
Sulphamerazine	*S. aureus* ..	45	40	400	300
	E. coli ..	40	45	400	400
	E. typhosa ..	40	45	400	400
Sulphaguanidine	*S. aureus* ..	300	250	300	300
	E. coli ..	300	250	250	200
	E. typhosa ..	400	400	300	200

TABLE III

Minimum inhibitory concentrations of surface-active compounds

Culture: *S. aureus*: 18 hrs., pH 7·6

Surface-active compound	Synthetic medium		Peptone medium	
	37°	43°	37°	43°
Temp. of incubation	mg. per cent.			
Nekal BX ..	12	12	10	12
Igepon T ..	20	30	24	30
Aerosol OT ..	5	8	24	30

No growth inhibition was observed against *E. coli* and *E. typhosa* with concentrations of surface-active agents less than 40 mg. per cent. in the synthetic medium and less than 200 mg. per cent. in the nutrient broth medium; higher concentrations were not tried. This is in accordance with the experience that anionic sufrace-active compounds of the usual type selectively inhibit the metabolism of Gram-positive organisms only.[35, 11]

As may be expected, greater amounts of the sulpha drugs are needed in peptone-broth medium than in the synthetic medium. This is also true of the surface-active compounds, especially Aerosol OT, against *S. aureus*. The interference due to peptone in the evaluation of detergents as disinfectants has been shown by others[36, 37, 14]; this may in part be due to phospholipids in peptone preparations which are known to neutralize the bacteriostatic activities of surface-active compounds.[38]

Differences between the drugs are also brought out better in the synthetic medium. There are variations in the order of their efficiencies at the two temperatures, in the two media and with the three organisms. Higher titres are needed against *E. coli* and *E. typhosa*.

While the concentration of drug for growth inhibition of the different organisms is more or less the same at the two temperatures of incubation, somewhat higher amounts of the surface-active compounds are needed at the higher temperature. With the rather limited data, no simple explanation is possible for this observation.

Synergistic effects of sulpha drugs and surface-active compounds.—With *S. aureus*, the wetting agents were employed in dilutions which in them-

selves were not bacteriostatic. The organism was then titrated against the sulpha drugs to ascertain the minimal concentrations for growth inhibition (Table IV). Synergism was inferred if the concentrations of the drugs were

TABLE IV

Synergism between sulpha drugs and surface-active compounds

Organism: *S. aureus*

Sulpha drug	Peptone-broth medium			Synthetic medium		
	Nekal BX 8 mg. %	Igepon T 20 mg. %	Aerosol OT 20 mg. %	Nekal BX 6 mg. %	Igepon T 10 mg. %	Aerosol OT 3 mg. %
	Milligrams per cent.					
Sulphanilamide	800*	800*	400	400	450	450
Sulphadiazine	100	100	20	10	10	12
Sulphapyridine	80	100	300*	15	10	15
Sulphathiazole	100	80	60	12·5	10	12·5
Sulphamerazine	100	80	20	30	25	35
Sulphaguanidine	300*	300*	300*	300*	250	300*

* No synergistic effect in these cases.

less than those given in Table II. With *E. coli* and *E. typhosa*, titrations were carried out against the surface-active compounds using sub-minimal amounts of the sulpha drugs. Some of the best combinations obtained for bacteriostasis are reported in Tables V and VI respectively.

TABLE V

Synergism between sulpha drugs and surface-active compounds

Organism: *E. coli*

Sulpha drug	Peptone-broth medium			Synthetic medium		
	Nekal BX	Igepon T	Aerosol OT	Nekal BX	Igepon T	Aerosol OT
	Milligrams per cent.					
Sulphanilamide	400+60	400+120	..	200+20	300+30	300+20
Sulphadiazine	300+40	200+ 80	300+80	12+20	12+25	10+30
Sulphapyridine	400+20	200+ 40	300+40	10+25	15+30	15+40
Sulphathiazole	200+20	100+ 40	200+40	10+20	12+30	12+20
Sulphamerazine	300+20	200+ 40	300+40	30+25	30+25	*
Sulphaguanidine	*	200+ 80	*	250+20	200+50	*

Figures in columns refer to sulpha drug and surface-active compound respectively.

* No synergism in these cases.

TABLE VI

Synergism between sulpha drugs and surface-active compounds

Organism: *E. typhosa*

Sulpha drug	Peptone-broth medium			Synthetic medium		
	Nekal BX	Igepon T	Aerosal OT	Nekal BX	Igepon T	Aerosol OT
	Milligrams per cent.					
Sulphanilamide ..	600 + 40	400 + 80	600 + 80	300 + 20	400 + 20	400 + 30
Sulphadiazine ..	200 + 40	200 + 80	200 + 80	12 + 20	12 + 25	12 + 20
Sulphapyridine ..	200 + 60	200 + 80	200 + 120	15 + 20	15 + 20	15 + 25
Sulphathiazole ..	200 + 20	100 + 40	100 + 40	15 + 30	15 + 20	10 + 20
Sulphamerazine ..	300 + 40	300 + 40	300 + 40	30 + 20	25 + 20	30 + 20
Sulphaguanidine ..	*	*	*	300 + 25	300 + 30	300 + 25

Figures in columns refer to sulpha drugs and surface-active compounds respectively.
* No synergism in these cases.

Tables IV–VI indicate marked synergism between drug and surface-active compound. This effect was least shown with sulphaguanidine, especially in peptone medium, against all the organisms. With the other sulpha compounds, the degree of synergism varied rather widely. While the surface-active compounds by themselves were ineffective against Gram-negative organisms, they had definite potentiating activity in conjunction with the sulpha drugs, the effect being more pronounced with peptone broth. The surface-active compounds apparently act both by facilitating drug penetration into the cell and by counteracting the natural sulphanilamide inhibitors. It has been demonstrated that Gram-negative micro-organisms can be sensitized to anionic surface-active compounds by means of protamine, possibly by its interaction with a natural inhibitory substance.[39] Munshi, *et al.*[22] have observed that under certain conditions surface-active anions might make a contribution to bacteriostasis by removing part of the essential metabolite, *p*-aminobenzoic acid, as a salt.

Additive effects of sulphanilamides and surface-active compounds on drug-fast organisms.—Resistant strains of *S. aureus* were obtained by successive transfers through increasing concentrations of sulphanilamide and sulphathiazole in peptone-broth medium. When tested against mixtures of sulphanilamide or sulphathiazole and Aerosol OT, growth inhibitions occurred at the same combined concentration of the two substances as with the original susceptible organism. Likewise, when *S. aureus* was grown in nutrient medium containing 0·2 per cent. of animal lecithin (Eastman Kodak) and

the cells washed and resuspended in nutrient broth before being used as inoculum, it was resistant to 30 mg. per cent. of Aerosol OT in nutrient medium (*cf.* ref. 38), but not to the same concentration of a mixture of sulphanilamide and Aerosol OT (Table IV) as the susceptible organism. Thus, resistance acquired to a sulpha drug or the inhibition of antibacterial effect of a surface-active compound is no longer manifested in presence of a combination of the two bacteriostatic agents. Treffers[40] has observed that a strain of *S. dysenteriae*, rendered resistant to penicillin, is not similarly resistant to a combination of penicillin and iodoacetic acid.

The present work has indicated that certain anionic surface-active compounds, besides potentiating the bacteriostatic properties of sulpha drugs, are effective against the acquirement of drug resistance. The effectiveness of the surface-active agents in combination with sulpha drugs against Gram-negative organisms would suggest that their bacteriostatic and surface-active properties are independent of each other. Whatever may be the mechanism of their synergising action, it would appear that, if non-toxic (*cf.* refs. 12, 41), they may find a place in sulphanilamide chemotherapy.

Summary

1. The bacteriostatic properties of sulphanilamide and five N^1-substituted sulphanilamides, and of three anionic surface-active compounds, have been studied, singly and together, against the organisms, *S. aureus*, *E. coli*, and *E. typhosa*, using peptone-broth and a synthetic medium.

2. With incubation temperatures of 37° and 43° the concentration of sulphanilamide for bacteriostasis in peptone medium is independent of the age of the culture and the concentration of the inoculum at the higher temperature only. In the synthetic medium, the effects of age of cells and size of inocula are less pronounced at both temperatures of incubation.

3. The minimum effective concentrations of sulpha drugs needed for growth inhibition are more in peptone broth than in the synthetic medium. Similar but less pronounced differences are seen with the surface-active compounds against *S. aureus*.

4. While the surface-active compounds are by themselves ineffective against the Gram-negative organisms, they have potentiating activity with the sulpha drugs.

5. The synergic effects of sulphanilamide and surface-active compound are unaltered even when the organism is rendered resistant to sulphanilamide or when the antibacterial property of the surface-active compound is neutralized by lecithin.

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