STUDIES ON HIGHLY SOLUBLE AZO SULFONAMIDES

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Since the early observations of Kellner (1936) and Fuller (1937), who demonstrated that *p*-aminobenzenesulfonamide (sulfanilamide) is formed in animal tissues when prontosil¹ or prontosil soluble² (neoprontosil³) is administered by mouth, and the studies by Domagk (1935), Colebrook and Kenney (1936), and Colebrook, Buttle, and O'Meara (1936), wherein they were unable to demonstrate an antibacterial effect on the part of the latter compounds *in vitro*, very little attention has been directed to similar studies on other azo sulfonamides. Inasmuch as several heterocyclic substituted sulfanilamide derivatives, namely, sulfathiazole, sulfapyridine, and sulfadiazine, have proved to be superior to sulfanilamide in their antibacterial effects against many organisms both *in vitro* and *in vivo*, it appeared worth while to compare the activity of these compounds with that of their azo sulfonamide counterparts.

An azo preparation with interesting properties, including the ability to inactivate lysozyme (Lawrence and Klingel, 1943), was prepared by coupling diazotized sulfathiazole with the symmetrical carbamide of 2R acid. The other soluble azo dyes were formed by coupling the diazotized parent sulfonamide compounds with sodium 1-hydroxy-7-acetylamino-naphthalene-3,6-disulfonate, hereafter described as "acetyl-2R acid." Diphenyl-sulfone-4,4'-disazo-acetyl-2R acid (a sulfone) and 6-sulfaquinoline-azo-acetyl-2R acid completed the series of drugs studied. The structural formulae and molecular weights of several of these compounds are given in figure 1.

The organisms against which the compounds were tested *in vitro* included *Staphylococcus aureus* 209, *Streptococcus pyogenes* C-203, a viridans streptococcus, types I, II, and III pneumococci, and members of the *Brucella* group.

IN VITRO METHODS

Studies on gram-positive cocci. Drug-broth solutions or suspensions of the compounds were prepared by adding the dry powders to 100-ml quantities of nutrient media contained in 250-ml cotton-plugged Erlenmeyer flasks. The test media, which also included a control lacking a drug, were autoclaved at 10 pounds for 10 minutes. Upon cooling, to each flask was added 1 ml of an 18-hour broth-diluted culture of one of the organisms.

The inoculated solutions were incubated at 37 C for 24 hours, after which time appropriate broth dilutions were made and 0.1-ml and 1.0-ml amounts were

¹ 4-Sulfamyl-2',4'-diaminobenzene hydrochloride.

² Sodium 1-hydroxy-2-(4-sulfamylbenzene-1'-azo)-7-acetylamino-naphthalene-6-disulfonate.

³ The registered trademark of Winthrop Chemical Company, Inc.

FIG. 1 STRUCTURAL FORMULAE AND MOLECULAR WEIGHTS OF SEVERAL AZO SULFONAMIDES

placed in petri dishes, and melted and cooled (45 C) nutrient agar was added. The contents of the plates were mixed thoroughly by swirling, and the agar was allowed to solidify. The plates were incubated at 37 C for 48 hours, following which period the growing colonies were counted.

Beef extract broth (pH 6.8, bacto peptone) and agar were used in most of the tests against the staphylococcus and viridans streptococcus. However, additional studies were included in which veal infusion broth (pH 7.4, bacto proteose peptone) containing 1 per cent sterile horse serum was used. Veal infusion broth containing glucose, as well as serum, was used in the tests with pneumococci and *S. pyogenes*. Sterile defibrinated rabbit blood was added to the veal glucose agar to promote growth of the latter organisms and to facilitate counting of the colonies.

In table 1 are presented typical results of the *in vitro* effects of 1 per cent concentrations of the several azo derivatives and their sulfonamide analogues upon *S. aureus* and the viridans streptococcus. In order to obtain more highly concentrated drug-broth solutions of the parent substituted sulfonamides (sulfathiazole, sulfapyridine, and sulfadiazine) for testing the organisms mentioned, these compounds were used in the form of their sodium salts. The pH of each solution or mixture in beef extract broth, as determined by the glass electrode potentiometer, is given in table 1.

In general, the two azo derivatives of sulfathiazole and the parent compound gave comparable degrees of bacteriostatic activity upon *S. aureus* in beef extract medium. This relative effectiveness was reduced considerably in the presence of 1 per cent serum when veal infusion broth was used. Although there appeared to be an inhibitory effect of the azo derivatives of sulfapyridine and sulfadiazine upon the staphylococci, this action was inferior to that exhibited by the parent compounds in corresponding concentrations. A similar correlation in effectiveness was noted between sulfanilamide and its azo derivatives. Sulfathiazole-azo-acetyl-2R acid was found to be the most active compound against viridans streptococci. This drug was followed in order of activity by sulfapyridine and sulfathiazole. The remaining compounds gave but a suggestion of a similar effect upon the latter organism.

Since the pH values of 1 per cent sodium salt solutions of the parent analogues of several of the compounds are somewhat higher than the pH of solutions of the corresponding azo derivatives, it would appear that some of the variations in antibacterial activities (table 1) could be attributed to the differences in pH. Furthermore the possibility that a partial breakdown of the soluble azo dyes to their corresponding active parent analogues may occur during autoclaving also seemed to merit consideration. Both of these points were studied simultaneously by first sterilizing concentrated aqueous solutions of all drugs by heating them in a boiling water bath 2 to 3 minutes, following which time the appropriate amount of the solutions was added to the sterile nutrient broth to give a final concentration of 1 per cent of the sulfonamides. A complete series of drug-broth and control broth media lacking a drug was adjusted to pH 7.80 (1 per cent sodium sulfathiazole in broth) and another to pH 8.90 (1 per cent sodium sulfapyridine in

broth) by the addition of sterile sodium hydroxide solution. With the use of *S. aureus* as the test organism in these studies, the differences in the degrees of activity of the compounds (table 2) were essentially the same as in table 1. In

TABLE 1

Effects, in vitro, of the compounds upon Staphylococcus aureus 209 and viridans streptococcus

COMPOUND	pH OF 1% DRUG- BROTH SOLUTIONS	1 PER CENT DRUG CONCENTRATIONS					
		<i>Staphylococcus aureus</i>			<i>Viridans streptococcus</i>		
		Beef extract broth		Veal broth 1% serum		Beef extract broth	
		Inoculum—organisms/ml					
		43	131	115	380	5,000	7,000
S	6.80	1.7*	3.6	0.26	90,000	23,000	29,000
S-Az	6.80	48,000	130,000	2,000	175,000	59,000	54,000
Na-ST	7.80	0.13	0.36	0.25	10,000	4,000	11,000
ST-Az	6.80	0.20	1.2	14	2,000	300	200
Na-SP	8.90†	4.8	15	0.50	60,000	2,000	3,000
SP-Az	6.85	53,000	25,000	10,000	75,000	69,000	67,000
Na-SD	7.30†	2,000	7,000	3,000	115,000	59,000	30,000
SD-Az	6.80	54,000	41,000	33,000	130,000	58,000	55,000
STC-Az	6.50	1.0	0.24	700	150,000	34,000	32,000
SQ-Az	6.70	2,000	27,000	1,000	115,000	86,000	86,000
D-Az	6.90	—	56,000	21,000	115,000	42,000	76,000
Control	6.90	118,000	335,000	125,000	180,000	84,000	76,000

Legend: S = sulfanilamide; S-Az = sulfanilamide-azo-acetyl-2R acid (prontosil soluble, "neoprontosil"); Na-ST = sodium sulfathiazole; ST-Az = sulfathiazole-azo-acetyl-2R acid; Na-SP = sodium sulfapyridine; SP-Az = sulfapyridine-azo-acetyl-2R acid; Na-SD = sodium sulfadiazine; SD-Az = sulfadiazine-azo-acetyl-2R acid; STC-Az = sulfathiazole-disazo-symmetrical-carbamide-2R acid; SQ-Az = 6-sulfaquinoline-azo-acetyl-2R acid; D-Az = diphenyl-sulfone-disazo-acetyl-2R acid.

* Figures, expressed in thousands (i.e., 1.7 = 1,700, etc.), represent number of cells per ml after 24 hours' incubation in broth + sulfa drug.

† Saturated solutions.

— = no test made.

subsequent tests similar results were obtained when unheated, sterile, Seitz-filtered solutions were used.

The effects of 5×10^{-4} molar (M/2,000) concentrations of the several compounds upon types I, II, and III pneumococci and *S. pyogenes* C-203 are summarized in table 3. These data indicate that molar equivalents of the two azo derivatives of sulfathiazole, 6-sulfaquinoline-azo-acetyl-2R acid, and sulfathiazole

itself were equally effective in inhibiting and, in some instances, in completely destroying the test organisms. Similar effects against the pneumococci were obtained with sulfapyridine, its corresponding azo derivatives, and diphenyl-sulfone-azo-acetyl-2R acid. However, a corresponding degree of activity upon *S. pyogenes* was not evidenced with the latter compounds. In general, sulfadiazine-azo-acetyl-2R acid was far inferior to sulfadiazine in inhibiting the

TABLE 2

Effects, in vitro, of the compounds upon Staphylococcus aureus in broth adjusted to various pH values

COMPOUND	pH	pH UNADJUSTED	pH ADJUSTED TO 7.80	pH ADJUSTED TO 8.90
		Inoculum—organisms/ml		
		130	116	52
S	6.80	3.6	12	3.6
S-Az	6.80	130,000	182,000	3,000
Na-ST	7.80	0.36	10	1.8
ST-Az	6.80	1.2	6.1	0
Na-SP	8.90†	15	90	7.4
SP-Az	6.85	25,000	63,000	20,000
Na-SD	7.30†	7,000	3,000	110
SD-Az	6.80	41,000	75,000	65,000
STC-Az	6.50	0.24	40	0
SQ-Az	6.70	27,000	16,000	—
D-Az	6.90	56,000	156,000	—
Control	6.80	335,000	675,000	540,000

See legend under table 1.

0 = no growth.

† Saturated solutions.

growth of this organism. A still greater divergency in the extent of bacteriostatic activity was noted between sulfanilamide and its corresponding azo derivative.

Studies on Brucella organisms. A different test procedure was used in studies on the *Brucella* organisms. A concentration of 1.0 per cent of the drugs was prepared in tryptose phosphate broth containing 0.1 per cent agar. Serial dilutions were made in the same medium to include dilutions of 1:200, 1:400, 1:800, and 1:1,600. After autoclaving, to each tube containing 5 ml of test solution was added a 4-mm loopful of a 24-hour broth culture of either *Brucella abortus*, *Brucella melitensis*, or *Brucella suis*. The tubes were incubated at 37 C and observed for visible growth at time intervals of 24, 48, and 72 hours. Tubes show-

TABLE 3
Effects, in vitro, of the compounds upon *pneumococci* and *Streptococcus pyogenes* C-203

COMPOUND	5 X 10 ⁻⁴ MOLAR (M/2,000) DRUG CONCENTRATIONS											
	Pneumococcus types											
	I		II		III		Streptococcus pyogenes C-203					
	Inoculum—organisms/ml											
	30	400	3	10	10	80			11	293		
S	132,000	20,000	2,500,000	15,000	0	4,000	117,000	565,000				
S-Az	1,000,000	290,000	400	113,000	600	1,000,000	480,000	405,000				
ST*	0.12	0.97	0	0	0	0	135	23,000				
ST-Az	0.24	0.69	0	0	0	0.44	260	15,000				
SP*	0.08	0.54	0	0	0	0.05	2,000	140,000				
SP-Az	0.51	0.94	0	0	0.44	2,000	46,000	390,000				
SD*	26	2	0	0.06	0	0.05	2,000	89,000				
SD-Az	470,000	1,000	0	1,000	7,000	16,000	53,000	625,000				
STC-Az	0.42	0.90	0	0	0	0.37	90	3,000				
SQ-Az	0.03	0.29	0	0	0.18	1.7	6.0	3,000				
D-Az	0.10	0.90	0	0	16	17,000	44,000	77,000				
Control	2,000,000	485,000	930,000	226,000	222,000	250,000	230,000	610,000				

See legend under table 1.

0 = no growth in plates inoculated with 0.1 ml of undiluted test mixture or dilutions thereof at the end of 24 and 48 hours' incubation of drug-broth-organism suspensions.

* Compounds were not used in the form of their sodium salts in this study.

ing no growth after 72 hours were tested for bactericidal action by transferring three 4-mm loopfuls to a tube of sterile tryptose phosphate agar broth. The results of this study are presented in table 4.

It will be observed that in general the azo derivatives compare in activity to equivalent weight concentrations of their respective parent sulfonamides in inhibiting the growth of the *Brucella* organisms. Broth dilutions of approximately 1:800 of all compounds proved to be bacteriostatic, and some were found to have a definite bactericidal effect against this group of gram-negative bacilli.

TABLE 4

Highest dilution of compounds showing antibacterial activity against Brucella organisms

COMPOUND	B. ABORTUS		B. MELITENSIS		B. SUIS	
	Bs	Bc	Bs	Bc	Bs	Bc
Na-S	1:400	1:400	1:800	1:400	1:1,600	1:200
S-Az	1:800	<1:100	1:400	<1:100	1:800	<1:100
Na-ST	1:1,600	1:800	1:1,600	1:400	1:800	1:400
ST-Az	1:1,600	1:200	1:800	1:200	1:200	<1:100
Na-SP*	1:400	1:400	1:800	1:400	1:1,600	1:200
SP-Az	1:400	<1:100	1:400	<1:100	1:400	<1:100
Na-SD	1:600	1:100	1:1,600	<1:100	1:800	<1:100
SD-Az	1:800	1:100	1:800	1:400	1:800	1:100
STC-Az	1:800	1:400	1:800	1:400	1:800	1:100
SQ-Az	1:800	<1:100	1:1,600	1:100	1:800	<1:100

See legend under table 1.

Na-S = sodium sulfanilamide.

Figures represent actual dilutions bacteriostatic (Bs) or bactericidal (Bc).

<1:100 = concentrations greater than 1:100 not tested.

IN VIVO METHODS

Chemotherapy of experimental streptococcal and pneumococcal infections in mice. Male albino mice weighing approximately 20 grams were inoculated intraperitoneally with 0.3 ml of a saline suspension of an 18-hour, veal glucose serum broth culture of either *S. pyogenes* C-203 or type II pneumococcus. The animals were medicated by means of a stomach tube 1½, 7, 12, 24, 32, and 48 hours after infection, and once daily thereafter until death occurred or up to and including the tenth day. The preparations were administered as a suspension or solution in 0.25 to 0.5 ml of milk. The results of studies in which sulfapyridine and its corresponding acetyl-2R acid derivative were used are presented in table 5.

It is apparent from the data given in table 5 that the sulfapyridine component of the azo sulfonamide is the portion of the molecule responsible for prolonging life or protecting the mice from experimental streptococcal and pneumococcal

infections; when gram equivalents of the two compounds are used, the parent sulfonamide is more effective in protecting the animals.

Chemotherapy of experimental Brucella abortus infections in mice. Kolmer (1940) tested five sulfonamides in the treatment of acute brucellosis in mice infected with *B. abortus* and obtained favorable therapeutic results with all of them. The compounds included sulfanilamide and its azo derivative neoprontosil. Both compounds appeared to be about equally effective in increasing the survival rate of infected mice. In our study the chemotherapeutic value of sulfadiazine and its azo derivative was compared in a similar brucellosis infection in mice.

TABLE 5

Chemotherapy of beta hemolytic streptococcus and type II pneumococcus infections in mice

ORGANISM AND COMPOUND	NUMBER MICE	DOSE		SURVIVAL IN HR		SURVIVAL: 10-DAY % OF SERIES
		Single mg/kg	Total g/kg	Range	Median	
Beta hemolytic streptococcus C-203						
Sulfapyridine	10	25	0.35	80-164	96	70
	10	100	1.40			100
	10	250	3.50			100
Sulfapyridine azo-acetyl-2R acid	10	25	0.35	21-240	53	20
	10	100	1.40	44-72	60	70
	10	250	3.50	220	220	90
Control—no drug	10			10-24	14	0
Type II pneumococcus						
Sulfapyridine	10	250	3.50	50-94	74	20
	10	500	7.00	48-196	78	20
	15	1,000	14.00	76-160	116	40
Sulfapyridine azo-acetyl- 2R acid	10	1,000	14.00	66-116	71	10
	10	2,000	28.00	68-240	91	40
Control—no drug	10			25-71	40	0

Male albino mice weighing approximately 20 grams were injected intraperitoneally with 0.2 ml of a saline suspension of a 24-hour tryptose phosphate agar slant culture of *B. abortus*. With the exception of one group of 10 mice, the animals were medicated by means of a stomach tube immediately after injection, 4 hours later, and twice each day thereafter for a total period of 14 days. The other group of mice was maintained on the same medication schedule, but the drug was administered by the subcutaneous route. The observation period continued for a total of 18 days. The drugs were administered as an aqueous solution or suspension.

Kolmer obtained his best therapeutic results against *B. abortus* infections in mice with the sodium formaldehyde sulfoxylate derivative of sulfanilamide,

which produced a survival of 56.2 per cent. In our tests sulfadiazine therapy showed a survival of 70 to 75 per cent, and the azo derivative of sulfadiazine

TABLE 6
Chemotherapy of Brucella abortus infections in mice

COMPOUND	NUMBER MICE	DOSE		SURVIVAL—HOURS		SURVIVAL— 18 DAYS % OF SERIES
		Single <i>mg/kg</i>	Total <i>mg/mouse</i>	Range	Median	
Sulfadiazine	20	100 oral	56	144-168	156	70.0
	20	50 oral	28	236	236	75.0
Sulfadiazine azo-acetyl- 2R acid	30	100 oral	56	236	236	80.0
	20	50 oral	28	96-120	108	80.0
	10	100 subq.	56	168	168	70.0
Control—no drug	30			96	96	23.3

TABLE 7
Effect of azo sulfonamides upon bacterial flora (E. coli) of intestinal tracts of mice

TEST DAY	DESOXYCHOLATE AGAR PLATE (E. COLI) COUNT GROUP NO. (REPRESENTING 5 MICE EACH)			
	1	2	3	4
3rd	Normal diet			
	I	H	I	I
	Sulfathiazole-azo-acetyl started on 3rd day		Sulfanilamide-azo-acetyl started on 3rd day	
	20	20	I	I
	0	0	I	I
5th 7th	Normal diet begun on 7th day			
	I	I	I	I
	I	T	I	I
	Sulfathiazole-azo-acetyl started on 13th day			
	—	—	0	0
16th 19th	—	—	0	0
	Normal diet begun on 19th day			
	—	—	T	I
	—	—	I	I
22nd	—	—	T	I
26th	—	—	I	I

Legend: I = innumerable (too many to count); T = thousands; H = hundreds; figures represent actual number of lactose-fermenting colonies in plate; 0 = no lactose-fermenting colonies observed; — = no test made.

caused a survival of 80 per cent. Oral medication of the azo compound appeared to be slightly more effective than subcutaneous administration.

Effects of azo sulfonamides upon intestinal coliform organisms in mice. Marshall *et al.* (1940) noted that the incorporation of sulfanilylguanidine in the diet of normal mice would cause a distinct reduction in the usual number of coliform organisms in the stools. Subsequent studies from our laboratories (Lawrence and Sprague, 1941) confirmed the observations of Marshall and his coworkers on sulfanilylguanidine. We noted, in addition, that sulfathiazole and sulfapyridine also caused a significant reduction of the number of coliform organisms normally present in the intestinal excreta of mice. Sulfanilamide, on the other hand, proved to be entirely ineffective in lowering the fecal *Escherichia coli* count of the animals.

In the present study several of the azo sulfonamides were tested for their ability to reduce the *E. coli* population in the stools of mice. A slight modification from the procedure used previously was made in the method for determining the effects of the compounds upon coliform organisms. Instead of plating fecal suspensions from each individual animal, approximately equal weights of stool specimens from each of a group of five animals were pooled, emulsified, and then plated in desoxycholate agar. Otherwise, the technique for estimating antibacterial actions of 1 per cent drug diets was the same as described before. Table 7 presents the results of a typical experiment using the azo derivatives of sulfanilamide and sulfathiazole. Note should be made of the observation that the azo compounds were excreted in the urine as well as in the feces as evidenced by the red pigmentation of the excreta.

From the data given in table 7 it will be noted that sulfathiazole azo-acetyl-2R acid caused a distinct lowering or complete disappearance of the *E. coli* flora of the medicated animals. The same attribute, however, was entirely lacking when the azo derivative of sulfanilamide was used. The data, furthermore, reveal that the same group of animals which failed to respond to the latter compound would, nevertheless, show a lowering of coliform organisms when placed on an azo sulfathiazole diet. Sulfadiazine azo-acetyl-2R acid gave results comparable to those obtained with the sulfathiazole derivative, whereas the azo analogue of sulfapyridine was somewhat less effective.

DISCUSSION

The limited solubility of the commonly known sulfonamides in aqueous solutions has resulted in the use of the sodium salts of the compounds when relatively high concentrations of the drugs are desired. The principal objection to the use of the sodium sulfonamides in wound therapy, irrigations, or in topical application has been the strong alkaline property of the compounds, with a pH of 10 or 11, which is caustic to tissues. Fletcher (1941) irrigated the antrums of a patient with sodium sulfathiazole and noted that the lining membrane was completely "charred" and destroyed. He attributed this toxic effect to the caustic action of the drug.

Adams (1943) recommended the use of sulfanilamide solutions in preference to sulfathiazole, sulfadiazine, etc., in disinfecting infected pulp and periapical lesions. He concluded from his studies that the neutral soluble derivatives of

the latter sulfonamides were objectionable, for they are so nearly insoluble that they persist in the tissues long enough to become foreign body irritants.

Wein *et al.* (1945) have recently described derivatives of sulfathiazole and sulfapyridine that are highly soluble, yet their local toxicity and irritant properties are less than those of the sodium salts of the sulfonamides. The compounds, "soluthiazole" and "solupyridine" are disodium cinnamylidene dibisulfite derivatives of the corresponding parent sulfonamides. They were found to be as active as the sodium salts of sulfathiazole and sulfapyridine in streptococcal and pneumococcal infections in mice. High concentrations of aqueous solutions of the solusulfonamides gave pH values of about 8.0.

Ten per cent solutions of many of the azo sulfonamides tested in our studies are readily obtainable by adding the powders to cold distilled water. In the concentration given, the sulfanilamide, sulfadiazine, sulfathiazole, and the sulfathiazole-symmetrical-carbamide derivatives give pH values of 5.3, 6.0, 3.7, and 7.1, respectively. The sulfapyridine and quinoline compounds are somewhat less soluble than the latter azo derivatives. One per cent aqueous solutions of the former two dyes have pH values of 6.70.

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SUMMARY

In vitro studies on several azo sulfonamides indicate that some of these soluble dyes compare favorably with their parent analogues in inhibiting the growth of gram-positive cocci. The azo derivatives of sulfathiazole appeared to be the most effective dye compounds tested with respect to their bacteriostatic and possible bactericidal actions on several of the organisms studied. Following, in order of diminishing activity, were the azo derivatives of sulfapyridine, sulfadiazine, and sulfanilamide.

Based on the assumption that the azo compounds owe their activity to the liberation in the animal body of the parent sulfonamides, evidence is presented to show that some of the azo derivatives are effective in protecting mice experimentally infected with pneumococci, streptococci, or *Brucella abortus*.

Incorporation of the heterocyclic, nonalkaline azo sulfonamides in the diet of normal mice resulted in a definite reduction in the coliform flora of their intestinal excreta.

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