THE INHIBITION OF THE BACTERIOSTATIC ACTION OF SULFONAMIDE DRUGS BY SUBSTANCES OF ANIMAL AND BACTERIAL ORIGIN*

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The inhibitory effect of peptone upon the bacteriostatic and bactericidal action of sulfanilamide was described by Lockwood (1). It was postulated that sulfanilamide may act upon microorganisms by preventing their utilization of protein-split products, and that peptone provided easily assimilable nitrogen in such excess that the bacteriostatic effect of sulfanilamide is decreased (2). Lockwood and Lynch (2) have recently extended this concept to explain the diminution of activity of sulfanilamide in the presence of purulent exudates, since under such circumstances the end-products of protein digestion may accumulate and interfere with the bacteriostatic action of the drug. Stamp (3) has described the presence of an inhibitory substance in hemolytic streptococci, and has partially purified inhibitor-containing extracts of these cells. Similarly, Green (4) has shown that an inhibitory substance may be extracted from organisms of the Brucella group, and Fleming (5) has demonstrated the presence of a sulfonamide inhibitor in heavy suspensions of staphylococcus, *Bacterium aertrycke*, *Streptococcus viridans*, and *Streptococcus pyogenes*.

The occurrence of the sulfonamide inhibitors is of importance in an understanding of the mode of action of this group of drugs both *in vitro* and in the animal body. For this reason a study has been made of the distribution of inhibitors in animal tissues and fluids, as well as in bacteriological media and bacterial cultures.

In order to test accurately for the presence of sulfonamide inhibitors it is necessary to use a culture medium which is free of these substances, and to choose an organism which will grow optimally in such a medium. *Bacterium coli* fulfills these requirements since it will grow luxuriantly in an inhibitor-free medium consisting of simple salts to which glucose is added as a source of carbon, and asparagin as a source of nitrogen. Sulfanilamide

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and sulfapyridine exert a pronounced bacteriostatic effect upon *B. coli* in this medium. Materials to be tested for the presence or absence of sulfonamide inhibitor may be added, and their effect determined more or less quantitatively. Moreover, the strain of *B. coli* used in these experiments produces relatively little inhibitor, so that the sensitivity of the test is therefore much increased.

In the present paper the occurrence of a substance or substances inhibiting the bacteriostatic effect of the sulfonamide drugs is described. It will be shown that the inhibiting substances occur in many but not all protein hydrolysates and that they are also present in certain normal body tissues. Furthermore, they have been found in sterile serous effusions, and are present uniformly in normal urine, although in this instance the inhibitory effect is readily demonstrable only after hydrolysis.

Materials and Methods

Method of Testing for Presence or Absence of Sulfonamide Inhibitors.—Tests for the presence or absence of sulfonamide inhibitors were performed by observing the effect of different amounts of the various materials upon the growth of *B. coli* in an inhibitor-free synthetic medium. To the medium was added an amount of sulfapyridine which in the absence of inhibitor, was sufficient to prevent the visible growth of the standard inoculum. Growth was estimated by observing the presence or absence of gross turbidity after incubation for 24 hours at 37°C. The synthetic medium to which sulfapyridine or the test material had not been added served as growth control in each experiment. In the presence of small amounts of inhibitor, maximum growth did not usually take place within 24 hours, so that estimation of the amount of inhibitor present could be made not only on the basis of the volume of test material added, but also on the amount of growth present at the end of the arbitrary test period. Furthermore, by varying the concentration of sulfapyridine, differences in the amount of inhibitor present could also be estimated.

Sulfapyridine.—A neutral solution of sulfapyridine was added to the synthetic medium. The final concentration varied in different experiments, and is indicated in each protocol.

Culture of B. coli.—A strain of *B. coli* in the smooth phase was used throughout. The stock culture was grown in the synthetic medium to which glycerol was added as a carbon source in place of glucose. It has been found that the organisms may be maintained in the smooth phase if cultivated in a glycerol-containing medium, whereas if glucose is used as the carbon source a mixed population of R and S forms develops on repeated subculture. The stock culture was stored in the ice box.

Inoculum.—The dilutions of the stock culture used in the tests were made in the synthetic medium. 10^{-4} cc. of culture was used to seed a volume of 5.0 cc. of culture fluid. This dilution contained between 70,000 and 100,000 cells, as determined by plate counts.

Culture Media: Synthetic Medium.—The synthetic medium used for the growth of

B. coli was that described by Sahyun, Beard, Schultz, Snow, and Cross (6), and contained the following ingredients:

Ammonium sulfate	4.72 gm.
Asparagin	2.0 "
Sodium chloride	5.0 "
Glucose	2.0 "
KH_2PO_4(M/5)	100.0 cc.
NaOH (N/1)	16.4 "
$MgCl_2$; $FeCl_2$; $CaCl_2$ (½ per cent mixture)	1.0 "
Distilled water	to 1000 "

The medium was sterilized in the autoclave; the pH was 7.6.

In the latter part of the work a casein hydrolysate known to be inhibitor-free, was added in the proportion of 2 gm. per liter of medium. The addition of the casein hydrolysate ensured an optimum growth rate. All readings of presence or absence of growth were made after 24 hours incubation at 37°C.

In the performance of bacteriostatic tests sulfapyridine in varying concentrations was added. The volume of medium inoculated with *B. coli* was 5.0 cc. in all instances. This volume included both the sulfapyridine and the solution to be tested for sulfonamide-inhibiting effect.

Liver Infusion Medium.—In order to test for the occurrence of sulfonamide inhibitor in the cells and culture supernatants of certain pathogenic bacteria, it was necessary to use an inhibitor-free medium which would support growth without the addition of peptone. Infusions of muscle were unsatisfactory both because of their content of inhibitor and also because growth of pneumococcus or *Streptococcus hemolyticus* did not occur unless a large inoculum was used. An infusion of fresh beef liver was found to be satisfactory, since it was free of inhibitor and supported the rapid and profuse growth of pneumococcus, *Streptococcus hemolyticus*, and staphylococcus following a minimal inoculum.

Calf's liver was obtained at the slaughterhouse as soon as possible after the death of the animal, and the gall bladder removed intact. The liver was placed immediately in boiling water for 5 to 10 minutes to destroy the autolytic ferments. It was then minced in a meat grinder and suspended in twice its weight of tap water including that in which it had been boiled. The pH was adjusted to 5.0 with HCl and the suspension heated in a water bath. The temperature was brought gradually to 80°C., at which it was maintained for 15 to 20 minutes. The supernatant fluid was decanted and filtered through coarse paper until clear. After cooling to room temperature solid sodium chloride was added to make a final concentration of 0.5 per cent and the pH adjusted to 7.8 with NaOH. A fine flocculent precipitate usually formed at this time which was removed by filtration through paper. The clear, fluorescent, yellow solution was sterilized by passage through Pasteur-Chamberland candles after first warming it to 35–40°C. to facilitate filtration.

This medium is somewhat unstable if kept at room temperature, but is more stable if stored in the dark and in the ice box. The addition in a final concentration of 2 per cent of either sterile ascitic fluid or fresh rabbit serum known to be inhibitor-free, was also found useful in stabilizing the growth-promoting properties of the medium.

The content of reducing sugars in different lots of this medium varied between 0.4 and 0.5 per cent.

It is important to use a culture medium which will support the optimum growth of the test organism since in a partially deficient medium the bacteriostatic effect of the sulfonamide drugs is greatly enhanced. Moreover, in a deficient medium the effect of materials containing a sulfonamide inhibitor may be extremely variable, since their action may be masked due to inadequate growth conditions.

EXPERIMENTAL

Presence of Sulfonamide Inhibitor in Bacteriological Culture Media.—The common bacteriological media used for the cultivation of pathogenic or-

TABLE I

Occurrence of Sulfonamide Inhibitor in Peptones and Casein Hydrolysates

Peptone or casein hydrolysate	Amount of preparation used	Sulfonamide inhibitor
	mg.	
Difco—bacto-tryptone	20	+++
" proteose peptone	20	+++
" bacto-peptone	20	+++
" neopeptone	20	+++
Fairchild's peptone	20	+++
Pfanstiehl's peptone	20	+++
Witte's peptone	20	Trace
Casein hydrolysate—enzymatic (Mead Johnson preparation No. 92Z38)	20	+++
Casein hydrolysate—acid	20	−
" " alkaline	20	−

Concentration of sulfapyridine: 1:20,000 in a final volume of 5 cc. of synthetic culture medium.

Test organism: *B. coli* in the smooth phase.

ganisms are prepared from infusions of muscle or parenchymatous organs and enriched by the addition of peptone. As originally noted by Lockwood (1) peptones are inhibitory to the bacteriostatic action of sulfanilamide on hemolytic streptococci. It was of interest, however, to determine whether the amount of inhibitor was the same in various brands of peptone and whether sulfonamide inhibitors were present in protein hydrolysates prepared by methods other than enzymatic degradation.

A series of commercially available peptones was tested for the presence of sulfonamide inhibitors. In addition enzymatic and acid and alkaline hydrolysates of casein were tested. In Table I the content of inhibitor in the various preparations is shown.

All of the peptones tested showed the presence of sulfonamide inhibitor. The enzymatic digest of casein contained a considerable amount of the

active substance, but hydrolysates of casein prepared by heating with concentrated acid or alkali were entirely free of inhibitor. It would appear, therefore, that sulfonamide inhibition does not depend upon the presence of easily assimilable nitrogen since the acid and alkaline hydrolysates of casein supply a great excess of nitrogenous substances and yet are entirely free of sulfonamide-inhibiting action. If an enzymatic casein hydrolysate which contains inhibitor is boiled under a reflux condenser with either concentrated acid or alkali, the inhibitor is destroyed, although it is resistant to boiling in dilute acid or alkaline solution.

Presence of Sulfonamide Inhibitor in Organs and Muscle.—The presence of sulfonamide inhibitor in various peptones and an enzymatic casein

TABLE II

Occurrence of Sulfonamide Inhibitor in Extracts of Fresh and Autolyzed Tissues

Tissue	Source	Sulfonamide inhibitor	
		Fresh tissue	Autolyzed tissue
Skeletal muscle	Rabbit	+	
Heart muscle	Beef	+	++++
Pancreas	"	++	++++
Spleen	Guinea pig	+	
"	Rabbit	+	
Liver	Beef	−	++++
"	Guinea pig	−	
"	Rabbit	−	
Kidney	"	−	

Concentration of sulfapyridine: 1:20,000 in a final volume of 5.0 cc. of synthetic culture medium. 1.0 cc. of the various tissue extracts tested is included in this volume.

Test organism: *B. coli* in the smooth phase.

hydrolysate and its absence from acid or alkaline hydrolysates suggested that the occurrence of this material is dependent upon enzymatic digestion. For this reason extracts of fresh organs and muscle from various species, as well as extracts of tissues which were permitted to autolyze under sterile conditions, were studied for the presence or absence of inhibitor.

Various organs and muscle were obtained from normal animals immediately following death, and boiled in tap water for 10 minutes to destroy the autolytic ferments. The boiled materials were minced in a meat grinder and extracted for 15 to 20 minutes in twice their weight of boiling tap water. The filtered extract, after the addition of NaCl to 0.5 per cent concentration, was adjusted to pH 7.6 and sterilized by boiling. Minced fresh tissues suspended in twice their weight of tap water were permitted to autolyze for 72 hours at 37°C. Toluene was added to prevent bacterial contamination. The autolyzed material was boiled and filtered, and the supernatant fluid tested for inhibitor after adjustment of the salt concentration and the pH. In Table II the

occurrence of sulfonamide inhibitor in various fresh and autolyzed tissues of certain animals is shown.

Extracts of fresh muscle, pancreas, and spleen as shown in Table II contained sulfonamide inhibitor. Fresh liver from three different species was entirely free of inhibitor, however. Rabbit kidney extract likewise was inhibitor-free. When autolysis had occurred under sterile conditions, the amount of inhibitor in extracts of muscle and pancreas showed a distinct increase, and the presence of the material could be demonstrated in autolyzed liver, although in the fresh state extracts of this tissue were entirely free of inhibiting effect.

Inasmuch as extracts of fresh muscle contain a sulfonamide inhibitor, and since inhibitor is present also in peptones, it is obvious that the common bacteriological media which contain these two ingredients are unsatisfactory for testing the bacteriostatic action of sulfonamide drugs. The widely divergent results of bacteriostatic tests reported by various workers might possibly be due to differences in the amount of inhibitor present in the various lots of media used, particularly since the amount of inhibitor varies from one peptone to another and also since any autolysis of the muscle used as the basis of the meat infusion has been shown to increase the amount of inhibitor.

The absence of the inhibiting material from extracts of fresh liver has proved of great advantage in that a bacteriological medium capable of supporting the luxuriant growth of certain fastidious microorganisms can be made from this organ. Pneumococci and hemolytic streptococci will grow readily in an infusion of fresh liver provided the infusion is not heated on the alkaline side. The addition of peptone is unnecessary.

Inhibitor-free liver infusion has been used as a culture medium for the study of the occurrence of sulfonamide inhibitor in bacteria and their growth products, and also to determine the relative sensitivity of certain strains of microorganisms to sulfanilamide or sulfapyridine. The occurrence of inhibitor in various bacterial cultures is described in a subsequent section of the present paper.

Occurrence of Inhibitor in Body Fluids.—The observation that a sulfonamide inhibitor is present in fresh normal organs and tissues suggested the possibility that a similar substance might be present in the fluids of the body.

Blood serum obtained during infection and in convalescence, and sterile effusions occurring in serous cavities during various diseases were tested for the presence or absence of inhibitor. The results of these tests are shown in Table III.

Sterile blood serum obtained from a total of sixteen patients ill with various diseases in no instance caused inhibition of the bacteriostatic effect of sulfapyridine under the conditions employed. On the other hand, sterile pleural effusions from two of twelve patients with acute lobar pneumonia showed the presence of inhibitor.[1] Pleural effusions from two patients with rheumatic fever contained inhibitor in both cases and in one patient suffering from chronic heart failure complicated by facial

TABLE III

Occurrence of Sulfonamide Inhibitor in Sterile Human Body Fluids

Body fluid	Clinical diagnosis	No. of cases	No. of specimens	No. of specimens showing presence of sulfonamide inhibitor
Blood serum	Acute lobar pneumonia	8	16	0
	Acute rheumatic fever	2	2	0
	Empyema	3	3	0
	Heart failure and erysipelas	1	1	0
	Carcinoma—mammary	2	2	0
Pleural fluid	Acute lobar pneumonia	12	12	2
	Acute rheumatic fever	2	2	2
	Heart failure and erysipelas	1	1	1
	Tuberculosis—pulmonary	1	1	0
	Nephrosis	1	1	0
Pericardial fluid	Acute rheumatic fever	1	1	1
Peritoneal fluid	Nephrosis	2	2	0
	Cirrhosis of liver	1	1	1
Knee joint fluid	Acute rheumatic fever	4	4	4

Concentration of sulfapyridine: 1:60,000 in a final volume of 5.0 cc. of synthetic culture medium. 1.0 cc. of the various fluids tested is included in this volume.

Test organism: *B. coli* in the smooth phase.

erysipelas, inhibitor was present in a pleural effusion. Peritoneal fluid from a patient with cirrhosis of the liver contained inhibitor. In one patient with acute rheumatic fever, the pericardial fluid showed the presence of sulfonamide inhibitor, and knee joint effusions obtained from four patients with rheumatic fever all exhibited marked inhibitory action. Peritoneal fluid obtained from two children suffering from so called "lipoid nephrosis" did not cause sulfonamide inhibition.

[1] We wish to thank Dr. Jesse G. M. Bullowa of Harlem Hospital, New York, for his generous assistance in obtaining specimens of pleural effusions, and blood serum.

It is of interest in relation to the data presented in Table III that serous effusions of the pericardium, pleura, and knee joint occurring during rheumatic fever, invariably showed the presence of sulfonamide inhibitor, whereas only two out of twelve pleural effusions occurring during lobar pneumonia were positive. The significance of these findings is as yet unknown.

Occurrence of Sulfonamide Inhibitor in Pus.—In the previous experiments the occurrence of inhibitor in sterile effusions was determined. In addition to these fluids, purulent exudates occurring in association with a number of infections have been tested. In all instances, boiled, cell-free extracts of pus have shown the presence of relatively large amounts of sulfonamide inhibitor. Pus has been obtained from staphylococcal and pneumococcal abscesses, empyemas of the pleura due to pneumococcus and an anaerobic streptococcus respectively, and in one case from pleural empyema from which no microorganism could be recovered. The presence of sulfonamide inhibitor in purulent exudates is not unexpected, since under these conditions considerable autolysis occurs, and as shown previously in the case of normal tissues, the amount of inhibitor increases greatly during the autolytic process.

Occurrence of Sulfonamide Inhibitor in Urine.—The occurrence of sulfonamide inhibitor in certain normal tissues and its absence from fresh liver, fresh kidney, and blood serum, suggested the possibility that the material might be excreted in the urine. Urine was collected from four normal individuals and from a patient suffering from empyema of the chest. Care was taken to prevent bacterial contamination.

The urine specimens were neutralized and boiled to ensure sterility. A portion of each specimen of urine was made acid with HCl in a final concentration of 0.2 N, placed in a narrow-necked flask to minimize contact with air, and boiled in a water bath for 45 minutes. The hydrolyzed urine specimens were neutralized, and again boiled in order to sterilize them. 1.0 cc. portions of each specimen were used in testing for the presence or absence of sulfonamide inhibitor. These data are presented in Table IV.

From the results shown in Table IV it would appear that most of the sulfonamide inhibitor is conjugated in the body and excreted in the urine in an inactive form. Of the five urines tested three did not show any inhibiting effect until acid hydrolysis of the urine was performed. The two specimens which contained only a trace of inhibitor before hydrolysis showed a marked increase after hydrolysis.

In addition to the above experiments with unconcentrated urine, in two instances concentrated urine was tested for the presence of inhibitor before and after acid hydrolysis.

Urine obtained from patients 1 and 2 (Table IV) was concentrated fiftyfold by evaporation in cellophane sacs suspended in a current of air. The concentrated urines were treated with 10 volumes of acetone in order to precipitate the excess salts present. The supernatant acetone solutions containing the inhibitor were taken down to dryness, and the dried material dissolved in water so that the final volume was one-fiftieth that of the original volume of urine. The solutions were brought to pH 7.6 and sterilized by boiling. A portion of each urine concentrate was then hydrolyzed in acid as described above for the unconcentrated specimens.

The concentrated urines were tested for sulfonamide inhibitor. In both cases before acid hydrolysis 1.0 cc. of urine concentrate, equivalent to 50.0 cc. of urine, did not cause inhibition of sulfapyridine 1:20,000.

TABLE IV

Occurrence of Sulfonamide Inhibitor in Urine before and after Acid Hydrolysis

Patient from whom urine was obtained	Sulfonamide inhibitor	
	Before acid hydrolysis	After acid hydrolysis
1. Empyema—sterile	−	+++
2. Normal	−	+++
3. "	−	+++
4. "	±	+++
5. "	±	++

Concentration of sulfapyridine: 1:60,000 in a final volume of 5.0 cc. of synthetic culture medium. 1.0 cc. of urine is included in this volume.

Test organism: *B. coli* in the smooth phase.

However, after acid hydrolysis 0.1 cc. of concentrate, equivalent to 5.0 cc. of urine, caused complete inhibition of sulfapyridine 1:20,000.

The observation that most of the sulfonamide inhibitor in urine is excreted in a conjugated form, suggested that the failure of liver and kidney extracts to inhibit might be due not to the absence of the inhibitor from these organs, but rather to its presence in an inactive, conjugated form. To test this possibility the following experiments were carried out.

Fresh rabbit liver was boiled to destroy the autolytic ferments. A portion of the finely minced tissue in tap water was brought to pH 1.5 by the addition of 4 N HCl. The other portion was kept at neutral reaction. Both suspensions were heated in a boiling water bath for 45 minutes. The pH was brought to 7.6 in each instance, and the volumes adjusted with tap water so that the amount of fluid was equivalent to twice the original weight of tissue. The suspensions were boiled again to ensure sterility and the liver tissue removed by centrifugation. The supernatant fluids were tested for their inhibiting effect. Fresh rabbit kidney was subjected to the same procedures and similarly tested. The results of these tests are shown in Table V.

From the observations recorded in Table V it would appear that inhibitor is actually present in both fresh rabbit liver and kidney, but that in these tissues it occurs in an inactive, conjugated form, since its effect is demonstrable only after hydrolysis. It is possible that the liberation of sulfonamide inhibitor during autolysis of liver may be due to enzymatic hydrolysis of conjugated inhibitor, in analogy to the release of the active form from liver and kidney upon acid hydrolysis. However, this does not rule out the possibility that inhibitor may be actually formed during autolysis or that more of preformed, active inhibitor may be released during cell breakdown than would result from extraction of intact, non-autolyzed cells.

TABLE V

Occurrence of Sulfonamide Inhibitor in Fresh Rabbit Liver and Kidney before and after Acid Hydrolysis

Organ extract	Sulfonamide inhibitor	
	Before acid hydrolysis	After acid hydrolysis
Rabbit liver	−	++++
" kidney	−	++++

Concentration of sulfapyridine: 1:20,000 in a final volume of 5.0 cc. of synthetic culture medium. 1.0 cc. of the various tissue extracts is included in this volume.

Test organism: *B. coli* in the smooth phase.

The finding of inhibitor in an inactive form in rabbit liver and kidney tissue affords an explanation for the occurrence of a similar material in urine. It seems likely that the inhibitor is conjugated in certain organs of the body and excreted in the urine in this form.

Occurrence of Sulfonamide Inhibitor in Bacterial Cultures.—The presence in bacterial cultures of substances inhibiting the bacteriostatic action of sulfanilamide or sulfapyridine has been described by several workers. Stamp (3) extracted hemolytic streptococci with dilute ammonia and demonstrated the presence of an inhibitor in these cells. Partial purification of the extracts was carried out. Similarly, Green (4) employing Stamp's technique succeeded in extracting a sulfonamide inhibitor from organisms of the Brucella group. This he termed a "pullulating factor" since some stimulation of bacterial growth occurred when partially purified extracts were added to bacterial cultures. In the opinion of this investigator, the action of the inhibitor is due to non-specific stimulation. Fleming (5) has described the inhibitory effect of suspensions of staphylococci, streptococci, and *B. aertrycke*, and considers that the antibacteriostatic

effect is due to the liberation of some substance or substances favoring bacterial multiplication. In addition to these observations on bacteria, Woods (7) and Woods and Fildes (8) have extracted from brewers' yeast an inhibitor of the sulfonamide drugs. The properties of a highly purified extract suggested to Woods and Fildes that *p*-aminobenzoic acid might act as a sulfonamide inhibitor. Tests with this substance showed it to have a powerful inhibiting effect, and Woods has suggested that this is the naturally occurring inhibitor present in bacteria and yeast. We have confirmed the observations of Woods and Fildes on the annulling effect of *p*-aminobenzoic acid on the bacteriostatic action of the sulfonamide drugs. Although many of the properties of the naturally occurring inhibitors are similar to those of *p*-aminobenzoic acid, it has been found that the inhibitors present in enzymatic digests of casein and in cultures of pneumococcus differ in certain of their chemical properties from *p*-aminobenzoic acid.

The occurrence of inhibitor in the bacterial cells and culture supernatants of strains of four bacterial species has been studied. The organisms used were *B. coli*, pneumococcus, staphylococcus, and a strain of *Streptococcus hemolyticus* belonging to the Lancefield group D.

The cultures of pneumococcus, staphylococcus, and streptococcus group D were grown in liver infusion; *B. coli* was grown in the synthetic medium. Both of these media are free of drug-inhibiting substances. In the cases in which the supernatant was used the cultures were boiled in a water bath and the cells removed by centrifugation. The supernatant liquid was brought to pH 7.6, 0.2 per cent dextrose was added, and the solution sterilized by boiling. Where suspensions of bacteria were used, the living cells were collected by centrifugation, washed twice in a large volume of M/15 phosphate buffer of pH 7.6, and resuspended in buffer. The washed cells were then heated in a boiling water bath. In addition to heat-killed pneumococci it was possible to test the inhibitory effect of washed suspensions of living pneumococci, since this organism is unable to multiply in the synthetic medium used for growing the test organism, *B. coli*. Furthermore, suspensions of living pneumococci were allowed to autolyze for 48 hours at pH 7.6 until only Gram-negative debris remained, and the clear autolysate after boiling was tested for inhibitory effect. Suspensions of living *B. coli* and the strain of streptococcus group D were extracted according to Stamp's method with N/25 ammonium hydroxide for 72 hours at 37°C. The extracts were centrifuged and the supernatants taken to dryness in a vacuum flask, dissolved in a small volume of water, brought to pH 7.6, and boiled. The drug-annulling effect of these preparations was tested by adding them to the synthetic medium containing sulfapyridine. *B. coli* was used as the test organism. The results of these experiments are shown in Table VI.

The data in Table VI demonstrate that in the various bacterial species the sulfonamide inhibitor shows differences in its distribution between the bacterial bodies and the culture fluid in which the cells have been

grown. In the case of *B. coli* grown on the synthetic medium, inhibitor is apparently restricted to the cells themselves and does not diffuse appreciably into the culture medium. Although washed, heat-killed *B. coli* do not cause inhibition, the presence of inhibitor in the cells can be demonstrated in extracts obtained by treating them with dilute ammonia. In

TABLE VI

Occurrence of Sulfonamide Inhibitor in Bacterial Cells and Culture Supernatants

Bacterium	Culture media*	Portion of culture tested	Amount of material used in terms of cc. of original culture	Concentration of sulfapyridine	Sulfonamide inhibitor
B. coli	Synthetic	Supernatant	5.0	1:60,000	−
		Cells:			
		1. Heat-killed	25.0	1:60,000	−
		2. Extracted with N/25 NH_4OH	150.0	1:20,000	++++
Staphylococcus aureus	Liver infusion	Supernatant	1.0	1:60,000	++++
		Cells:			
		Heat-killed	30.0	1:60,000	−
Pneumococcus Type I	Liver infusion	Supernatant	1.0	1:60,000	++
		Cells:			
		1. Living	7.5	1:20,000	−
		2. Heat-killed	7.5	1:20,000	−
		3. Autolyzed	75.0	1:20,000	−
Streptococcus group D (Lancefield)	Liver infusion	Supernatant	1.0	1:60,000	−
		Cells:			
		1. Heat-killed	30.0	1:60,000	++
		2. Extracted with N/25 NH_4OH	150.0	1:20,000	++

The final volume of synthetic culture medium used was 5.0 cc. in each instance.

Test organism: *B. coli* in the smooth phase.

* The culture media used to grow the four strains of bacteria were free of inhibitor before inoculation.

the case of the strain of streptococcus group D, a naturally occurring sulfonamide-resistant strain, the inhibitor is likewise restricted to the cells and the supernatant is free of inhibitor under the conditions of these experiments. On the other hand, *Staphylococcus aureus* and Pneumococcus Type I released into the medium amounts of inhibitor which were easily demonstrable, whereas the cells themselves were inhibitor-free. Extraction of the cell bodies with dilute ammonia was not carried out in these instances. It is of interest that suspensions of washed, living pneumococcal

cells did not produce inhibitor in a medium in which this organism is unable to grow, and that the completely autolyzed cells from a large volume of culture were free of inhibitor.

Difference in the Amount of Sulfonamide Inhibitor Produced by Sulfapyridine-Susceptible and Sulfapyridine-Fast Strains of Pneumococcus Type I. —We have described previously certain alterations which take place in cultures of Pneumococcus Type I upon the acquisition of fastness to sulfapyridine (9). These changes include a greatly diminished production of hydrogen peroxide on the part of the sulfapyridine-fast strain, as well as a diminution in the activity of its dehydrogenase systems toward glycerol, lactate, and pyruvate. No change in type specificity or virulence accompanied the metabolic alterations. It was of interest to determine whether or not the sulfapyridine-fast strain differs from the susceptible parent strain in the production of sulfonamide inhibitor. Cultures of the two strains of Pneumococcus Type I were grown in the liver infusion medium. The bacterial cells and supernatant culture medium were tested for inhibitor. The results of these tests are shown in Table VII.

TABLE VII

Comparative Amounts of Sulfonamide Inhibitor in Cultures of Parent and Sulfapyridine-Fast Strains of Pneumococcus Type I Grown in Inhibitor-Free Liver Infusion Medium

Strain of Pneumococcus Type I	Portion of culture tested	Amount of material used in terms of cc. of original culture	Concentration of sulfapyridine	Sulfonamide inhibitor
Parent strain	Supernatant	1.0	1:60,000	++
		0.6	1:60,000	−
		0.2	1:60,000	−
Sulfapyridine-fast strain	Supernatant	1.0	1:60,000	++++
		0.6	1:60,000	++++
		0.2	1:60,000	++++
Parent strain	Cells:			
	1. Living	7.5	1:20,000	−
	2. Heat-killed	7.5	1:20,000	−
	3. Autolyzed	75.0	1:20,000	−
Sulfapyridine-fast strain	Cells:			
	1. Living	7.5	1:20,000	±
	2. Heat-killed	7.5	1:20,000	−
	3. Autolyzed	75.0	1:20,000	−

The final volume of culture medium was 5.0 cc. in each instance.
Test organism: *B. coli* in the smooth phase.

The results shown in Table VII make it clear that the acquisition of sulfapyridine fastness by a strain of Pneumococcus Type I is accompanied by a great increase in the production of sulfonamide inhibitor. 1.0 cc. of supernatant of the parent culture inhibits only partially the bacteriostatic effect of sulfapyridine in a concentration of 1:60,000 whereas 0.2 cc. of culture supernatant of the fast strain completely inhibits the same concentration of sulfapyridine. The cell bodies of the two strains of pneumococcus do not differ significantly in their inhibiting effect. Whether the increased amount of sulfonamide inhibitor produced by the sulfapyridine-fast strain is causally related to the various other metabolic changes previously described (9) is not known. An understanding of this relationship must await more complete analysis of the metabolism of the two strains of pneumococcus.

DISCUSSION

The widespread distribution of a substance or substances which annul the bacteriostatic action of the sulfonamide drugs is of importance not only in relation to the use of these chemotherapeutic agents in the treatment of infections but also in relation to the mode of action of the drugs upon bacterial cells *in vitro*. In an important contribution to this subject Woods (7) has demonstrated that *p*-aminobenzoic acid annuls the bacteriostatic effect of sulfanilamide *in vitro*, and that the inhibition is not due to a molecular reaction between *p*-aminobenzoic acid and sulfanilamide. Woods has postulated that *p*-aminobenzoic acid is essential for the growth of bacteria, and that sulfanilamide causes bacteriostasis by inhibiting the enzyme reaction involved in the utilization of *p*-aminobenzoic acid. As a corollary to this theory, Woods has suggested that differences in the sensitivity of bacteria to sulfanilamide may be due to quantitative differences in their ability to synthesize *p*-aminobenzoic acid. Selbie (10) has shown that *p*-aminobenzoic acid, administered by mouth, inhibits the curative action of sulfanilamide in experimental infections of mice with group A hemolytic streptococci.

Certain of the properties of the sulfonamide inhibitors occurring in enzymatic hydrolysates of casein and in culture supernatants of pneumococcus are similar to those of *p*-aminobenzoic acid. However, an important difference exists in their solubilities in acid ether. *p*-Aminobenzoic acid may be extracted readily by ether from aqueous solutions at pH values below 4.5. However, the inhibitors from these natural sources are only slightly extractable under the same conditions. Woods has pointed out that novocaine, which is the hydrochloride of the *p*-aminobenzoic ester of *N*-diethyl-amino-ethyl alcohol [$H_2N \cdot C_6H_4 \cdot CO \cdot OCH_2 \cdot CH_2N(C_2H_5)_2$] is also

a very potent sulfonamide inhibitor. In respect to solubility in acid ether, novocaine behaves somewhat similarly to the inhibitors from casein digests and pneumococcal cultures in that it is only slightly soluble. This is to be expected since the esterification of *p*-aminobenzoic acid removes its acidic properties. According to Woods the inhibitory effect of novocaine is slightly delayed, hence it may be possible that hydrolysis of the ester linkage is necessary before this compound becomes active. This investigator has stated that the properties of *p*-aminobenzoic acid and the inhibiting material obtained from yeast show many similarities. However, he is of the opinion that the yeast factor may be different from *p*-aminobenzoic acid, although its structure and properties are similar.

In the present paper the occurrence of active inhibitors in certain normal animal tissues is described. It is also shown that in rabbit liver and kidney tissue and in human urine, most of the inhibitor is present in a conjugated form so that its activity becomes evident only after acid hydrolysis. Harrow, Power, and Sherwin (11) have shown that when *p*-aminobenzoic acid is fed to either man or rabbit, part of it is excreted as *p*-acetylaminobenzoic acid. In a later paper Harrow, Mazur, and Sherwin (12) suggest that in addition to the excretion of an acetyl derivative of *p*-aminobenzoic acid, a portion may also be excreted as a glucuronate. In the present study the finding of an inactive, conjugated derivative of the sulfonamide inhibitor in rabbit kidney, liver, and in human urine, suggests that the inhibitor is handled by the body in a manner similar to that by which *p*-aminobenzoic acid is metabolized.

Whatever the nature of the substance or substances responsible for sulfonamide inhibition, their importance in relation to the therapeutic use of the sulfonamide drugs should be borne in mind. The presence of an inhibitor in many of the tissues and fluids of the body as well as in association with the invading bacteria affords an explanation for the occurrence of localized lesions, resistant to the action of the sulfonamide drugs, which may develop during the course of therapy even though necrosis or cell autolysis is not demonstrable. Thus for example, it is conceivable that empyema may be more likely to develop during the treatment of pneumococcal pneumonia with sulfapyridine if an initially sterile effusion contains sulfonamide inhibitor, than if such an effusion is free of inhibitor.

In the study of the effect of the sulfonamide drugs upon bacteria *in vitro*, it is important that a medium initially free of inhibitor should be used, otherwise comparison of the results of different experiments with the same test organism is not possible. The commonly used bacteriological media are not satisfactory since both the peptone and tissue infusion used in their preparation are known to vary from lot to lot in their content of

inhibitor. Moreover, different strains of the same bacterial species may vary in their susceptibility to the sulfonamide drugs, and as has been shown in the case of Pneumococcus Type I a greatly increased amount of inhibitor is produced when sulfapyridine fastness develops.

SUMMARY

Sulfonamide inhibitor has been demonstrated in extracts of fresh normal muscle, pancreas, and spleen of certain animals. When autolysis of tissues takes place the amount of inhibitor is greatly increased.

Fresh liver from beef, rabbit, and guinea pig is free of active inhibitor, although inhibitor is demonstrable in autolysates of this tissue. Fresh rabbit kidney is likewise free of active inhibitor. Following acid hydrolysis extracts of fresh rabbit liver and kidney cause sulfonamide inhibition.

Normal human urine contains little or no active inhibitor. However, upon acid hydrolysis, inhibitor is uniformly present.

Sulfonamide inhibitor is present in some, but not all, sterile serous effusions occurring during certain diseases. Inhibitor was found uniformly in pus. None was found in blood serum.

In certain species of bacteria the inhibitor is found in the cells only and is not demonstrable in the culture medium, whereas in other species, the inhibitor is found in the culture supernatant, and the cells themselves are relatively free.

The development of sulfapyridine fastness in a strain of Pneumococcus Type I is accompanied by a greatly increased production of sulfonamide inhibitor.

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