THE RELATIONSHIP OF SULFAPYRIDINE, NICOTINIC ACID, AND COENZYMES TO THE GROWTH OF STAPHYLOCOCCUS AUREUS

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A number of hypotheses have been offered in explanation of the mode of action of sulfanilamide and allied compounds (1–8). However, the problem is so complex that no one hypothesis is in itself satisfactory. Our studies have indicated that one of the important rôles of these compounds is their effect on the metabolism of the bacterial cell. In trying to define this effect more closely, we have investigated the relationships between these drugs, growth substances, and the multiplication of microorganisms. The purpose of this paper is to present observations on some of these relationships which represent one facet of a many sided problem.

Preliminary observations showed that effective bacteriostasis by sulfanilamide was not a result of its changing the culture medium. Effectiveness appeared dependent on its modifying the bacterium. Hemolytic streptococcus grown in sulfanilamide or in sulfapyridine for 6 hours and then washed until the supernatant fluid was free of drug was unable to multiply in normal manner on transplantation to broth containing the same compound. This was less striking when the incubation was for 4 hours and still less when for only 2 hours. These observations were in accord with those of Chandler and Janeway (9). All of our evidence indicated that organisms were modified by adequate concentrations of the drug. However, existing methods were not sufficiently delicate to detect any disappearance of the drug from the medium. Crossley (10) meanwhile has demonstrated that a small amount of the drug combines with the microorganism and that the combination cannot be broken by repeated washings.

The union of drug and microorganisms is unfavorable to the growth of the bacterium. However, this adverse effect is not necessarily a permanent one. Organisms which survived for 16 hours or longer in sulfanilamide or sulfapyridine showed rapid growth on transplantation to media containing these drugs. As seen in Fig. 1, organisms which survived for over 16 hours

in sulfanilamide multiplied almost as rapidly in sulfanilamide broth as in normal broth. These organisms had become "fast." This development suggested that variation might be possible in a bacterial enzyme system whereby the organisms, temporarily incapacitated by the drug, could adapt themselves to the new environment. The concept that sulfonamides may affect a bacterial enzyme system conforms with one of the striking characteristics of the action of the drug *in vitro*—that is, the lag. As Chandler and Janeway (9) and McIntosh and Whitby (11) have pointed out, growth occurs normally for at least 5 hours in the presence of these compounds. In striking contrast to the germicides, sulfanilamide does not modify the growth curve until 6 hours after inoculation. This failure of sulfanilamide to show a bactericidal effect before 6 hours suggested that certain essential requirements for bacterial growth might be supplied initially by intracellular sources, as did the fact that the addition of cellular debris (12, 13) to the medium nullified the bacteriostatic effects of these drugs. Similarly, Stamp (14) has clearly shown that a fraction of a broth culture of hemolytic streptococcus antagonizes the bacteriostatic action of sulfanilamide and suggested that this fraction may contain a necessary nutritive factor against which the drug is active. Recently Green (15) has described a growth-stimulating antisulfanilamide factor obtained from *Brucella abortus*. He feels that his "P" factor may stimulate the same enzyme reaction in the bacterial cell which sulfanilamide inhibits. Finally, Woods (16) has

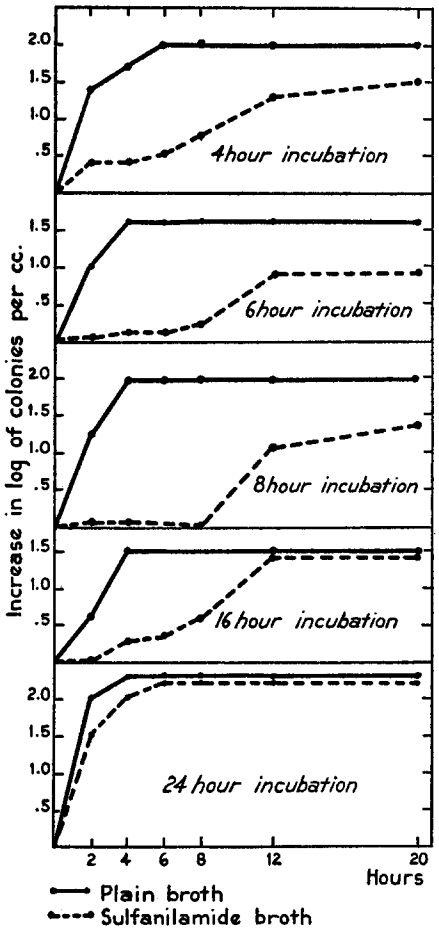

FIG. 1. Growth of *Streptococcus hemolyticus* with and without sulfanilamide following preliminary incubation in sulfanilamide broth for various periods of time (4 to 24 hours).

observed that *p*-aminobenzoic acid neutralizes the bacteriostatic effect of sulfanilamide.

The fact that nicotinic acid amide and sulfanilamide and sulfapyridine contain certain similar chemical groupings, the appearance of porphyrinuria on adding sulfonamide compounds to (17) or withdrawing nicotinic acid from the diet (18), and the inhibition by sulfapyridine of lactic and pyruvic acid dehydrogenase (19, 20) in the pneumococcus all suggested that sulfapyridine may inhibit the synthesis or proper functioning of coenzyme I or II. It therefore seemed advisable to study the effect of sulfapyridine, nicotinic acid, and coenzymes on an organism for which nicotinic acid was essential for growth.

Materials

Medium.—The basal medium was made of Nelson's photographic gelatin No. 1, imported from England by the Eastman Kodak Company. Care was exercised to remove all traces of barium from the hydrolysate. The medium was sterilized by autoclaving. Knight's procedure (21) was followed throughout, with the exception that thiamine was not used.

Organism.—A strain of hemolytic *Staphylococcus aureus* recently isolated from the throat was employed.

Chemicals.—Nicotinic acid was obtained from the Eastman Kodak Company, the sodium salt of sulfapyridine and highly purified yeast coenzymes (A Co value 132,300) from Merck and Company.

Crude coenzymes were prepared by centrifuging citrated normal human blood (22), discarding the plasma, diluting the packed red cells with three volumes of distilled water, heating on a boiling water bath until the proteins were coagulated, and filtering. The filtrate was then adjusted to pH 4.7 with N/1 hydrochloric acid, again heated, and a second small coagulum removed by filtration. Half of the extract was adjusted to pH 6.5 with N/1 NaOH and sterilized by boiling on the water bath for a few minutes; the other half was adjusted to pH 10 (just blue to thymol blue) and autoclaved at 120°C. for 30 minutes to destroy coenzymes.

The highly purified yeast coenzymes were dissolved in water and half of the solution was sterilized for 10 minutes in a boiling water bath; the other half inactivated by autoclaving as described above, a little Na_2HPO_4 being used instead of NaOH. Plasma extracts were prepared exactly as were red cell extracts.

All water used was triple distilled, and all glassware, including pipettes and test tubes, were cleaned with bichromate sulfuric acid cleaning fluid, washed thoroughly with triple distilled water, and sterilized by autoclaving. Nicotinic acid and sodium sulfapyridine were sterilized by passing through a Seitz filter.

Procedure

All tubes containing 10 cc. of basal medium were inoculated from a 24 hour culture grown on an agar slant. A heavy suspension of organisms was diluted until the final suspension contained less than 1,000 colonies per cc. Clouding of the medium, plating

with a wire loop, and pour plating when indicated were used to observe growth. Incubation was at 37°C. for 48 hours. When the colonies were too numerous to count, they are recorded as + to ++++. The numbers in the tables indicate the colonies per cc. of Knight's medium.

RESULTS

Effect on Growth Curve of Nicotinic Acid Added to Basal Medium

Table I shows that the organism was unable to multiply on the basal medium until nicotinic acid was added, confirming Knight (21).

TABLE I

Hours	0	4	6	8	10	12	14	16	24
Basal medium	614	304	188	105	111	124	125	107	278
Basal 0.1 cc. M/100 nicotinic	672	248	208	198	+	++	+++	++++	++++
Basal 0.1 cc. M/1,000 nicotinic	118	18	32	19	83	5,516	5,120	+	+++

TABLE II

Basal medium	Nicotinic acid M/1,000	Sulfapyridine M/1,000	Hours					
			0	14	16	18	20	24
cc.	*cc.*	*cc.*						
10	0.1	0	74	3,000	26,000	30,000	+	++
10	0.1	0.15	104	0	10	10	110	0
10	0.1	0	69	17,000	30,000	72,000	++	++++
10	0.1	0.15	148	0	0	0	0	0

Effect of Sulfapyridine and Nicotinic Acid on Growth Curve in Basal Medium

This experiment was designed to determine whether sulfapyridine exerted its bacteriostatic effect in the presence of nicotinic acid. The results of two typical experiments are presented in Table II.

It is seen that the bacteriostatic action of sulfapyridine is exerted in the presence of nicotinic acid, under these experimental conditions.

Effect of Sulfapyridine and Red Blood Cell Extract on Growth Curve in Basal Medium

Since the bacteriostatic effect of sulfapyridine was not inhibited by free nicotinic acid, the effect of nicotinic acid chemically combined as coenzymes was next investigated. As a convenient source of coenzymes, human red blood cell extract was used, autoclaved extract acting as a control.

The red cell extract as made was of such a concentration that 1.5 cc. had to be added to each tube of basal medium to correspond to 0.15 cc. of M/1,000 coenzyme solution (22). It is recorded in the latter way in Table III in order to conform to the other tables.

The sulfapyridine failed to inhibit growth in the presence of active red cell extract, but was effective in doing so in the presence of autoclaved extract. Since coenzymes are present in red cell extract and are destroyed by autoclaving in weak alkali, we investigated the possibility that this effect was due to coenzymes.

TABLE III

Basal medium	Active red cell extract M/1,000	Inactive autoclaved red cell extract M/1,000	Sulfapyridine M/1,000	Hours					
				0	14	16	18	20	24
cc.	*cc.*	*cc.*	*cc.*						
10	0.15	0	0.10	106	±	+	++++	++++	++++
10	0.15	0	0.10	115	±	+	++++	++++	++++
10	0	0.15	0.10	73	0	0	0	0	0
10	0	0.15	0.10	90	0	0	0	0	0

Effect of Sulfapyridine and Blood Plasma Extract on Growth Curve in Basal Medium

As plasma does not contain coenzymes, an extract was prepared similar to that made from red blood cells. Our experiments showed that these extracts also inhibited the bacteriostatic effect of sulfapyridine. However, the inhibition persisted when extracts autoclaved at pH 10 were used. This was in sharp contrast to the red cell extract and suggested that substances other than coenzymes were responsible for the effect. Since the results with the plasma extracts were not always constant, tables are omitted.

Effect of Sulfapyridine and Crude Yeast Extract on Growth Curve in Basal Medium

As yeast is a rich source of coenzymes, a crude extract of pressed bakers' yeast was prepared by the same methods used in making the red blood cell extracts. The crude yeast extracts, both fresh and autoclaved, inhibited the bacteriostatic effect of the sulfapyridine, suggesting that there were other substances in addition to coenzymes responsible for this inhibition. These yeast extracts, though rich in coenzymes, were not sufficiently purified for the study of coenzyme effects alone.

Effect of Sulfapyridine and Highly Purified Yeast Coenzymes on Growth Curve in Basal Medium

As the preceding experiment indicated that crude yeast extract contained interfering substances, it was repeated with highly purified yeast coenzymes (A Co value 132,300). Colony counts were not done in this experiment. The tubes were inoculated, clouding observed, and streak cultures made on blood agar plates after 24 and 48 hours are recorded in Table IV.

The highly purified yeast coenzymes inhibit the bacteriostatic effect of sulfapyridine, while the autoclaved control does not do so.

TABLE IV

Basal medium	Sulfapyridine M/10	Active coenzymes M/500	Inactive autoclaved coenzymes M/500	Hours	
				24	48
cc.	*cc.*	*cc.*	*cc.*		
10	0.1	0.2	0	+++	++++
10	0.1	0	0.2	±	0

DISCUSSION

It is evident that substances other than coenzymes inhibit the bacteriostatic effect of sulfapyridine. Our observations show that, under the conditions of these experiments, coenzyme interferes with the bacteriostatic action of sulfapyridine; whereas nicotinic acid fails to have this effect. A possible explanation is that sulfapyridine and nicotinic acid compete for the same position in the coenzyme molecule. Given nicotinic acid, the organism on the basal medium is presumably able to form coenzymes (nicotinic acid amide adenine dinucleotide). However, given nicotinic acid and sulfapyridine, the organism may be unable to form coenzymes or the activity of certain dehydrogenases (coenzymes combined with protein) may be inhibited. When preformed coenzymes are present in the medium, the normal metabolism of the organism is not modified by sulfapyridine and the customary growth curve is seen. As all cultures were aerobic, it seems improbable that coenzymes functioned merely as reducing agents.

SUMMARY

Our studies indicate that sulfapyridine modifies the normal metabolism of a bacterium.

Coenzymes inhibit the effect of sulfapyridine on the growth of staphylococcus in Knight's medium.

Nicotinic acid does not interfere with the action of sulfapyridine under the same conditions.

The possible relation of sulfapyridine, nicotinic acid, and coenzymes in relation to bacterial growth is discussed.

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