NON-ACETYLATION OF SULFANILAMIDE DURING ACQUIREMENT OF DRUG RESISTANCE IN *BACILLUS SUBTILIS*

BY SUDHA JOSHI AND A. SREENIVASAN, F.A.SC.
(*Department of Chemical Technology, University of Bombay*)

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THERE is a possibility that the acquisition of resistance to a drug may be due to the development by the organism of an ability to detoxify the drug. Thus, Woolley (1944) observed that resistance to pyrithiamine in *Endomyces vernalis* was accompanied by the adaptive formation of an enzyme which split pyrithiamine into two non-toxic parts. The presence of penicillinase in certain penicillin-resistant *Staphylococci* has been demonstrated by several investigators (Abraham and Chain, 1940; Kirby, 1944; Bondi and Dietz, 1945; Gots, 1945).

Other instances of drug resistance involving synthesis of an enzyme elaborated for the destruction of the drug do not appear to have been examined in detail. There are indications however that such mechanisms may exist. Miller and Bohnhoff (1944) noted a type of streptomycin resistance in *Meningococci* wherein the organism required streptomycin in the medium for its growth. Emerson and Cushing (1946) reported a sulfonamide-resistant strain of *Neurospora* for which sulfanilamide (SA) acted as a growth factor; it was presumed that enzyme(s) for converting sulfanilamide into some essential metabolite were developed in the organism.

Acetylation of sulfonamides decreases their growth inhibitory effect (Wyss, Strandskov, Schmelkes, 1942). An enzyme system that could acetylate sulfonamides among other amino compounds has been recognized in animal tissues (Marshall, Cutting and Emerson, 1937; Fuller, 1937) and is now known to be associated with co-enzyme A (Lipmann, Kaplan, Novelli, Tuttle and Guirard, 1947). Novelli and Lipmann (1947) have shown the ability of bacteria to convert pantothenic acid into Coenzyme A. With *Staphylococcus aureus*, Green and Sevag (1944) observed that sulfonamides interfere with the functions of pantothenic acid and riboflavin. Pantothenic acid was also found to reverse the effect of sulfonamides (Green and Sevag, 1944; Kersey and Porter, 1948). Miller, Bruno and Berglund (1947) observed an increase in the synthesis of pantothenic acid in *Escherichia coli*

on acquisition of resistance to sulfonamides. SA-resistant strains of *E. coli* reportedly average about 30 per cent. higher in Coenzyme A content than the parent sensitive ones and N^4-acetyl SA has no effect on growth of *E. coli* in a synthetic glucose-salt medium (Kayser and Metzinger, 1954).

The probable critical role of pantothenic acid in sulfonamide metabolism, its involvement as Coenzyme A in the acetylation of sulfonamides and the reduction by it of the growth inhibitory effect of sulfonamides, all suggested a possibility that organisms might acquire resistance by developing a capacity to detoxify the drug through acetylation which would imply increased elaboration of the acetylating enzyme.

The present studies were aimed at ascertaining if sulfonamide detoxification by acetylation is effected enzymatically by the drug-resistant organism.

Experimental

All studies reported were carried out with a local isolate of *Bacillus subtilis*. Resistance to SA (500 mg. per cent.) was induced by exposure to gradually increasing concentrations of the drug in the growth medium. The synthetic medium of Muir, Shamleffer and Jones (1942) supplemented with one part per hundred of a 10 per cent. acid hydrolysate of casein was used. Cultures were maintained at 37° on slants of this medium containing 2 per cent. agar with fortnightly transfers.

The organism was grown in bacteriological test-tubes containing 5 ml. quantities of double strength medium with additions, where shown in the text, final volume being made to 10 ml. before sterilization at 15 lb. steam pressure for 15 minutes and inoculation. Incubations were for stated periods. Necessary unincubated controls were always run alongside.

SA was determined by the method of Bratton and Marshall (1939), colour intensity being measured on a Klett-Summerson photo-colorimeter at 540 mμ. Acetylated SA was deduced from the difference between free and total SA, the latter being determined after hydrolysis with 0·5 ml. of 4 N hydrochloric acid in boiling water-bath.

Estimations were made in bacteria-free culture filtrates deproteinised with trichloroacetic acid to final concentration of 4 per cent. Values reported represent averages of at least duplicate determinations.

Results

Acetylation of SA during growth.—The extent of acetylation of SA during growth was studied with the susceptible parent strain as well as with

strains rendered resistant to 50 and 500 mg. per cent. respectively of SA. The influence of added pantothenic acid and methionine as accessory factors was also studied. Methionine has been observed (unpublished data) to stimulate pantothenic acid synthesis in organisms like *E. coli* and *B. subtilis* that can grow independently of the vitamin. Additions of methionine and pantothenic acid were tried analogously with observations of Bellamy and Gunsalus (1944) that tyrosine decarboxylase is produced in optimal amounts by *Streptococci* only when pyridoxine, nicotinic acid and tyrosine are present in the medium. Results are given in Table I.

TABLE I

Acetylation of SA in culture filtrates

Supplements to 10 ml. of medium + sulfanilamide*	Susceptible strain		Strain resistant to 50 mg. per cent. SA		Strain resistant to 500 mg. per cent. SA	
	At 0 hours	After 72 hours' growth	At 0 hours	After 48 hours' growth	At 0 hours	After 48 hours' growth
	Acetyl SA in μg.					
None ..	20	60	..	20	..	20
Calcium pantothenate (200 μg.)	80	80	80	50	Nil	40
Methionine (200 μg.) ..	40	60	30	50	30	50

*Essentially similar results were obtained using concentrations of SA up to 2–5 mg. per tube.

With the susceptible strain, growth was scanty in presence of the drug and hence a longer incubation period was employed. The resistant strains gave good growth within 18 hours. Free and total SA was also estimated in these cases at the end of 24 hours and 72 hours but no acetylation could be observed.

With the strain resistant to the higher concentration of SA, it was observed that a diazotizable arylamine was produced in the culture filtrate in considerable quantities even in the absence of added SA. Thus, under the conditions of the foregoing experiments, this arylamine formed amounted to 240 μg. and did not increase on acid hydrolysis. Control tubes sterilized

after inoculation and incubated simultaneously for various periods of time did not show presence of the arylamine. Further studies on this arylamine formation are reported in a subsequent communication (Joshi and Sreenivasan, in press).

Acetylation of SA by respiring cells.—Large quantities of cells were harvested in 100 ml. of the media in Roux bottles at 37° for 18 hours. The cells were centrifuged, washed with M/5 phosphate buffer (pH 7·4) till free from SA and resuspended in 25 ml. of the buffer. 5 ml. aliquots of this suspension were taken in 8 ml. of the buffer and allowed to respire in presence of small quantities of SA at 37° for 20 hours, the total volume being adjusted to 20 ml. Both free and total SA after acid hydrolysis were estimated before and after incubation for the specified period. The effects of sodium acetate, sodium citrate and accessory additions like pantothenic acid and methionine were also studied. The results with the susceptible strain are given in Table II.

TABLE II

Acetylation of SA by resting cells

Additions	Free SA (μg.)		Total SA (μg.)	
	0 Hours	20 Hours	0 Hours	20 Hours
Control (cells in buffer + added SA)	198	198	200	202
Na-acetate (1 M) + Na-citrate (0·5 M)	200	202	200	202
Ca pantothenate (100 μg.) + Methionine (100 μg.).. ..	198	200	198	198

There is no acetylation of SA by respiring cells of the susceptible strain even in presence of sodium acetate and sodium citrate. Calcium pantothenate and methionine also have no influence. Concentrations of SA up to 5 mg. per tube were tried without any effect.

Similar results were obtained with respiring cells of 0·05 per cent. and 0·5 per cent. SA- resistant strains; in the latter case, there was also no appreciable production of the arylamine during respiration.

It has been shown by Novelli and Lipmann (1950) that when yeast cells are enriched by incubation with glucose and pantothenic acid in phosphate buffer, acetate and glucose metabolism is increased and there is an increase in Coenzyme A formation. In a few experiments, cells of 0·5 per cent. SA-resistant strain of the organisms, washed free of the drug, were suspended in 5 ml. of 0·1 M phosphate buffer, pH 7·4, containing glucose (1 M) and calcium pantothenate (2 mg.) and aerated for 1 hour. The cells were separated by centrifugation and washed twice with the buffer. Five ml. aliquots of a suspension of these cells in 25 ml. of the buffer were allowed to respire for 20 hours in 0·2 M phosphate buffer containing varying concentrations of SA with and without calcium pantothenate (200 μg.) in a total volume of 10 ml. In no case however any indication of acetylation of SA was obtained. Enrichment with various concentrations of glucose and pantothenate was also tried without any effect.

DISCUSSION

SA is not acetylated to any appreciable extent by susceptible or SA-resistant strains of *B. subtilis* during growth or under respiring conditions. SA was employed in these experiments as it is known to be acetylated to a greater extent than *p*-aminobenzoic acid, sulfathiazole or sulfadiazine. Detoxification of sulfonamides through acetylation does not therefore appear to be the mechanism of drug resistance in *B. subtilis*.

There are reports to suggest that acetylation is only a subsidiary function of Coenzyme A. This may be the reason why, in spite of the increased synthesis of pantothenic acid reported by Miller *et al.* (1947) in *E. coli* under the action of sulfonamides, acetylation does not occur and is not therefore the cause for sulfonamide resistance. The data further show that the added drug remains unchanged under various experimental conditions. It seems therefore that no adaptive enzyme is formed for detoxification or utilization of SA along with acquisition of drug resistance.

A diazotizable arylamine is formed in the culture filtrate of 0·5 per cent. SA-resistant strain of *B. subtilis*. Further studies on this arylamine formation are reported in a subsequent communication (Joshi and Sreenivasan, in press).

SUMMARY

Cells of susceptible and sulfanilamide-resistant strains of *Bacillus subtilis* were harvested under various cultural conditions and examined for their ability, if any, to acetylate sulfanilamide.

The effects of added acetate, citrate and accessory factors such as pantothenic acid and methionine were studied.

Experiments were also carried out employing cells enriched with glucose, phosphate and pantothenic acid.

The results showed no measurable acetylation of sulfanilamide in all cases.

A diazotizable arylamine accumulated in the culture filtrates of 0·5 per cent. sulfanilamide-resistant strain, but not of the susceptible strain, during growth of the organism and even in the absence of added sulfanilamide.

It is concluded that detoxification of sulfonamides through acetylation is not the mechanism of drug resistance in *B. subtilis*. There is also no adaptive enzyme formation for the detoxification or utilization of sulfanilamide during acquisition of drug resistance.

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