MECHANISM OF RESISTANCE TO 5-AZACYTIDINE IN *Bacillus subtilis*. II.* UTILIZATION OF 5-AZACYTIDINE AND OF NATURAL PYRIMIDINES

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A comparative study of the utilization of 5-azacytidine in a sensitive and a resistant strain of *Bacillus subtilis* revealed fundamental differences concerning incorporation of 5-azacytidine-4-^{14}C into nucleic acids. In the 5-AzCyd-s** strain the incorporation of the analogue is high while in the 5-AzCyd-r strain no incorporation of the analogue into either type of nucleic acid takes place. The transport of the antimetabolite across the cell membrane from a minimal medium without glucose is by 60% lower in *B. subtilis* 5-AzCyd-r than in 5-AzCyd-s. The conclusion that the passage of this compound across the membrane may play a principal role in the mechanism of resistance is supported by the finding that in the presence of 5-azacytidine-[4-^{14}C] in a medium with glucose the sensitive cells can take up and maintain a high level of overall radioactivity-^{14}C whereas the levels of radioactivity in the resistant strain are negligible. The utilization of natural precursors of nucleic acids, cytidine, uridine, and thymidine, is not substantially affected in the 5-AzCyd-r strain.

Investigation of the resistance to 5-azacytidine extends our knowledge of the mechanism of inhibitory action of this antimetabolite. In previous papers we described especially the anabolic transformation of 5-azacytidine[1] which is characterized by a pronounced antileukemic[2] and bacteriostatic[3] effects. The anabolic conversion of 5-azacytidine and incorporation of 5'-polyphosphates of this analogue into mammalian[5], plant[6], bacterial[7,8] and phage [9]nucleic acids has been demonstrated. Its application to mice affects primarily lymphoid tissues and bone marrow where it inhibits the synthesis of nucleic acids[4]. Of the inhibitory phenomena *in vitro*, the effect of 5-azacytidine on RNA synthesis in isolated cell nuclei of calf thymus should be mentioned here[10]. When studying the changes associated with the formation of resistance to 5-azacytidine in mouse leukemic cells, a substantially reduced uridine-kinase

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** Abbreviations used: 5-AzCyd-s and 5-AzCyd-r- sensitivity and resistance, respectively, of strains to 5-azacytidine; DNA deoxyribonucleic acid; RNA ribonucleic acid; TCA trichloroacetic acid; cyd, thd, urd cytidine, thymidine and uridine; SSC 0·15M-NaCl and 0·015M sodium citrate; CMP, CDP, CTP cytidine -mono-, di- and triphosphate; UMP, UDP, UTP uridine mono-, di-, and triphosphate; C, 5-MC, U, T cytosine, 5-methylcytosine, uracil, and thymine.

activity[11] as well as a drop of incorporation of 5-azacytidine into RNA was observed[12]. Present communication describes the changes in the utilization of pyrimidines and of 5-azacytidine due to resistance to this analogue in *Bacillus subtilis*. Only results obtained *in vivo* are presented.

EXPERIMENTAL

5-*Azacytidine*-[4-^{14}C] (4 mCi/mmol) was prepared at the Department of organic synthesis and in the Isotope laboratory of this Institute. Cytidine (^{3}H or ^{14}C), uridine (^{3}H or ^{14}C) and thymidine-[^{14}C] were obtained from the Institute for Research, Production and Application of Radioisotopes, Prague. Nucleosides of natural pyrimidines were from Koch-Light (Great Britain).

Bacterial cultures and incubation media. For all the experiments we used cultures of *Bacillus subtilis* SMYW 5-AzCyd-s or 5-AzCyd-r. Both prototrophic[13] strains grew under agitation at 37°C in a minimal Spizizen medium[14] enriched with 1% Vitamin Free-Casamino Acids (Difco) and 0·5% glucose. Only when following short-term penetration of 5-azacytidine into cells we used a buffered solution of 0·15M-NaCl with 0·015M sodium citrate (pH 7), designated as SSC in the following. Growth was evaluated according to turbidity at 570 nm in a Kaucký spectrophotometer.

To determine the incorporation of natural precursors into nucleic acids, the method of Schneider was used in principle[15]. Culture samples were withdrawn into equal volumes of 20% TCA precooled to 0°C, left for 15 min in ice and further treated by the membrane-filter technique with washing by 20 ml of cold 5% TCA. To determine the incorporation into DNA, aliquots of the cultures were pipetted into an equal volume of 0·6N-KOH, left for 18 h at room temperature, cooled to 0°C and, after adding 50% TCA to a final concentration of 10%, they were treated analogously on membrane filters. The values of incorporation into RNA were obtained by substracting the values for incorporation into DNA from the sum of incorporation.

Total radioactivity in cells after application of 5-azacytidine-[4-^{14}C] was determined by membrane-filter technique with SSC used for washing.

Isolation of nucleic acids. To determine the incorporation of the antimetabolite which is unstable at extreme pH values[16] we used simplified isolation procedures. To isolate RNA we used a culture washed several times with buffered SSC. After treatment with lysozyme (200 μg/ml 15 min, 37°C) the RNA was extracted with ice-cold 0·01M sodium acetate of pH 5 with 0·5% sodium lauryl sulfate and an equal volume of water-saturated phenol containing 0·001M ethylenediaminetetraacetic acid and 0·1% 8-hydroxyquinoline. The mixture was agitated for 2 min at 0—4°C. After a ten-minutes centrifugation at 10 000 r.p.m., the extraction of the phenol layer and of the intermediate phase was repeated twice. RNA was precipitated from the combined supernatants with 2·5 volumes of ethanol containing 2% potassium acetate at —18°C overnight. Samples of RNA for radioactivity measurements were dissolved in SSC.

Isolation of DNA was carried out by a simplified method according to Marmur[17]. The lysozyme-treated bacterial pellet was deproteinized with chloroform–isoamyl alcohol (24 : 1). After centrifugation for 15 min at 10 000 r.p.m. the DNA was precipitated from the supernatant with 2 volumes of ethanol. After dissolving the fibrous precipitate, pancreatic ribonuclease (a solution heated for 10 min to 80°C at pH 5) was added to a final concentration of 100 μg/ml and the solution was incubated for 15 min at 37°C. After incubation, an equal volume of water-saturated phenol was added and the mixture was vigorously shaken for 30 min. The intermediate layer and the phenol phase were extracted once more after centrifugation (15 min, 10 000 r.p.m.). DNA was precipitated from the pooled supernatants with 2 volumes of ethanol.

The level of free nucleotides was determined after application of labelled cytidine or uridine in culture during an early exponential phase of growth. The bacteria were washed several times with physiological saline and the sediment after centrifugation (10 min at 5 000 r.p.m.) at 4°C was suspended in a small amount of distilled water and frozen to −30°C. The bacterial suspension was frozen and thawed several times and finally, under constant agitation, cooled TCA was added to a final concentration of 10% and the samples left in ice for 30 min. After centrifugation, TCA was extracted from the supernatant with ether and 50 mg deactivated charcoal (Brilonite 4 n) was added to the aqueous layer. The mixture was acidified with acetic acid to a final concentration of 0·01 M. After 2 h of shaking the charcoal was put on a small column and the adsorbed substances were eluted with a 50% solution of spectrally pure ethanol containing 0·5% ammonia. The individual nucleotides were separated by chromatography on Whatman No 1 in isobutyric acid–water–ammonia (66 : 33 : 1·5). Zones absorbing UV light in the nucleotide region (according to standards) were eluted and hydrolyzed in concentrated $HClO_4$ (105°C, 1 h)[18]. After hydrolysis the bases were identified by chromatography on Whatman No 1 in isopropyl alcohol–HCl–water (170 : 41 : 39)[19].

Specific labelling of pyrimidine bases of RNA *and* DNA, after application of labelled cytidine or uridine, was carried out in samples treated in the same way as during incorporation of natural pyrimidines into nucleic acids. The sediments after precipitation of samples with cold TCA and 70% ethanol were hydrolyzed with concentrated $HClO_4$ and chromatographed for identification of bases as shown above.

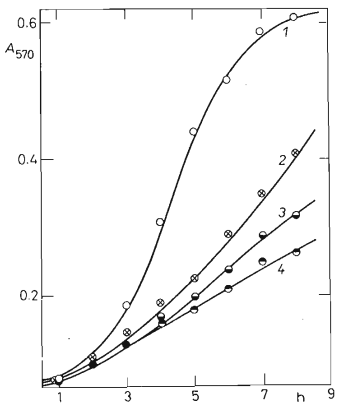

FIG. 1

Effect of 5-Azacytidine on the Growth of *Bacillus subtilis* Sensitive to 5-Azacytidine

Cultures of *B. subtilis* grew in a minimal Spizizen medium enriched with 1% vitamin-free casamino acids (Difco) and 0·5% glucose. E_{575} growth, *h* time of cultivation. *1* Control, *2* 5-Az.-Cyd 2·5 μg/ml, *3* 5-AzCyd 25 μg/ml, *4* 5-AzCyd 100 μg/ml.

Radioactivity was assayed according to the nature of the samples either in a scintillation counter Packard Tricarb (model 3375) in a toluene scintillation solution (4 g 2,5-diphenyloxazol and 250 mg 1,4-bis(2-phenyloxazolyl)benzene per liter of toluene) or on aluminium planchets in an automatic flow counter (Frieseke-Hoepfner).

RESULTS

The effect of 5-azacytidine on the growth of cells sensitive to 5-azacytidine is depicted in Fig. 1. In the sensitive strain, even the lowest concentration used ($5 . 10^{-5}$M) shows an inhibitory effect. The growth of the resistant strain proceeds unaffected at the same concentrations of the antimetabolite. Of the isolated 5-AzCyd-r mutants[13] we used in all experiments only one type, designated as R_0.

In further experiments we studied the incorporation of 5-azacytidine into nucleic acids and its transport across the cell membrane. The incorporation of the antimetabolite is depicted in Fig. 2. No incorporation into DNA or RNA of the resistant

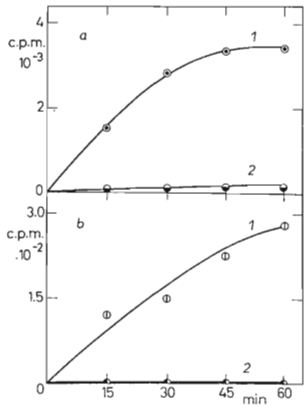

FIG. 2

Incorporation of 5-Azacytidine into Nucleic Acids of *Bacillus subtilis* Sensitive and Resistant to 5-Azacytidine

Values of incorporation were obtained from 10 ml bacterial culture for RNA and from 20 ml culture for DNA, both A_{575} 0,25. Min time of incubation. Concentration of 5-azacytidine-[4-^{14}C] (specific activity 4 mCi/mmol) was 0·1 μCi/ml culture. *a* incorporation into RNA; *b* incorporation into DNA. *1* 5-AzCyd-s, *2* 5-AzCyd-r.

strain was demonstrated while under the same conditions the sensitive strain displays a relatively high incorporation into both nucleic acids. Incorporation in the 5-AzCyd-s strain rises with time and keeps to be linear for 30 min. These differences were confirmed even when assaying the total radioactivity in cells (Fig. 3*a*). It follows from the comparison of total radioactivities in 5-AzCyd-s and 5-AzCyd-r that in the cells of the resistant strain no inhibitory effects may be expected even at the level of lowmolecular precursors of nucleic acids since no form of radioactivity, was detected in this strain. These results led us to the investigation of the passage of 5-azacytidine across the cell membrane (Fig. 3*b*). Under conditions when a change of the incubation medium suppressed the possibility of phosphorylation and incorporation of 5-azacytidine while the transport could continue we observed a 60% decrease of penetration of 5-azacytidine into the resistant strain as compared with the sensitive one.

In some cases of resistance one may compare the metabolic changes of utilization of natural precursors to assess the nature of the mechanism of resistance to the

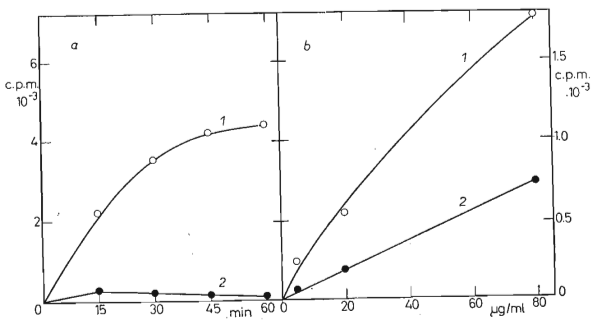

FIG. 3

Total Radioactivity in *Bacillus subtilis* Cells Sensitive and Resistant to 5-Azacytidine after Application of 5-Azacytidine-[4-^{14}C]

a Cells for determination of total radioactivity grew for different periods (min) in a minimal Spizizen medium enriched with 1% vitamin-free casamino acids (Difco) and 0·5% glucose. The values of radioactivity hold for 1 ml bacterial culture, A_{575} 0,25 at zero time. Applied radioactivity of 5-azacytidine-[4-^{14}C] was 0·1 μCi/ml medium (specific activity 4 mCi/mmol time of incubation min). *b* Transport of 5-azacytidine across the cell membrane was evaluated from bacterial culture A_{575} 0·3, 1 ml samples of bacteria were preincubated for 15 min without the antimetabolite, then incubated for 30 min, at 37°C in the presence of various concentrations of 5-azacytidine in a minimal Spizizen medium without organic supplements. Abscissa concentration of 5-azacytidine in 1 ml.

analogues of nucleic acid bases[20]. Therefore, we studied further the incorporation of natural pyrimidine nucleosides into nucleic acids of both strains (Fig. 4). It follows from the figure that in this case the acquirement of resistance does not result in substantial changes in the ability of cells to incorporate cytidine, uridine and thymidine into nucleic acids. Incorporation experiments were accompanied by measuring the total radioactivity in cells of both strains. This comparison also shows that the utilization of pyrimidines is not substantially affected. The total radioactivity lower by 10% and radioactivity in nucleic acids lower by 20% observed with resistant mutant after the administration of cytidine-[^{14}C] cannot be held responsible for the resistence.

The results obtained during the incorporation of natural nucleosides are in agreement with those for free nucleotides in the cells of both strains (Table I). After application of cytidine-[^{3}H] we did not find any differences in the levels of its phosphorylated products. In the same experiment with uridine-[^{3}H] we observed a 16% shift of radioactivity from cytidine mononucleotide to cytidine polyphosphates in the resistant strain.

From the point of view of anabolic conversion we thought it useful to determine the specific labelling of pyrimidine bases of nucleic acids after application of cyti-

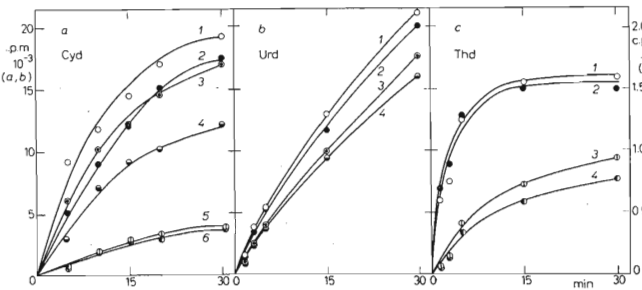

FIG. 4

Total Radioactivity in Cells and Incorporation into Nucleic Acids after Application of Natural Pyrimidine Nucleosides in Strains of *Bacillus subtilis* Sensitive and Resistant to 5-Azacytidine

Values of radioactivity hold for 1 ml bacterial culture A_{575} 0·25 at zero time which grew in a minimal Spizizen medium enriched with 1% vitamin-free casamino acids (Difco) and 0·5% glucose. Uridine-U-[^{14}C], cytidine-U-[^{14}C], and thymidine-2-[^{14}C] were applied at 0·1 μCi/ml medium and 2·5 μg/ml. Time of incubation min. *1 a b c* total radioactivity in cells of 5-AzCyd-s after application of the nucleoside shown in the figure; 2 A B C total radioactivity in cells of 5-AzCyd-r; *3 a* incorporation of cytidine into RNA of 5-AzCyd-s; *4 a* incorporation of cytidine into RNA of 5-AzCyd-r; *5, 6 a* incorporation of cytidine into DNA of 5-AzCyd-s and 5-AzCyd-r; *3, 4 b* incorporation of uridine into RNA of 5-AzCyd-s and 5-AzCyd-r; *3, 4 c* incorporation of thymidine into DNA of 5-AzCyd-s and 5-AzCyd-r.

dine-[^{3}H] (Table II) and the effect of excess uridine on cytidine incorporation into nucleic acids (Fig. 5) of both strains. The results of these experiments showed that the anabolism of cytidine proceeds identically in both strains and that it thus plays no role in the mechanism of resistance.

TABLE I

Level of Free Pyrimidine Nucleotides in *Bacillus subtilis* Sensitive and Resistant to 5-Azacytidine

Values shown in % were obtained from 20 ml bacterial culture A_{575} 0·2. Uridine-U-[^{3}H] and cytidine-U-[^{3}H] were used at 1·0 μCi/ml culture at a final concentration of 1·5 μg/ml. The samples were incubated for 30 min at 37°C. Details see Experimental.

B. subtilis strain	Radioactivity in free nucleotides, %				Total c.p.m.	
	(UTP + UDP)	UMP	(CTP + CDP)	CMP	U	C
			Uridine-[^{3}H]			
5-AzCyd-s	74	26	29	71	2 345	2 360
5-AzCyd-r	73	27	44·5	55·5	2 405	2 700
			Cytidine-[^{3}H]			
5-AzCyd-s	0	0	32	68	0	1 060
5-AzCyd-r	0	0	31·5	68·5	0	1 130

TABLE II

Incorporation of Cytidine into Pyrimidine Bases of Nucleic Acids in *Bacillus subtilis* Strains Sensitive and Resistant to 5-Azacytidine

The values shown in % were obtained from 10 ml bacterial culture $A_{575} = 0·2$. Cytidine-U-[^{14}C] was used at 0·08 μCi/mu culture at a final concentration of 2·5 μg/ml. The samples were incubated for 30 min at 37°C. Details see Experimental.

B. subtilis strain	Radioactivity in nucleic acid pyrimidines, %			Total c.p.m.
	C + 5-MC	U	T	
		RNA		
5-AzCyd-s	50	50	0	60·200
5-AzCyd-r	41	59	0	53·700
		DNA		
5-AzCyd-s	52·5	0	47·5	13·400
5-AzCyd-r	53	0	47	17·570

DISCUSSION

In the formation of drug resistance in bacteria, mammalian cell cultures or neoplastic cells, three from genetic point of view fundamental mechanisms play a role: phenotypic adaptation, induced mutation and selection of pre-existing resistant cells[21,22]. One of the biochemical mechanisms of its manifestation is the decreased penetration of inhibitory substances across the cell membrane[23]. This possibility was considered in this work.

The decreased penetration of 5-azacytidine across the cell membrane in 5-AzCyd-r, as compared with sensitive cells, indicates stereospecific structural changes in the cell membrane. One might also envisage a mutation-caused change in the primary structure of the permease which is responsible for the transport of the antimetabolite across the membrane. A direct proof of this possibility would require different experimental approaches, including an isolation of the purified enzyme preparation which has not been achieved so far. From the point of view of quantitative participation of the cell membrane in the mechanism of resistance it is important to note the difference between the total radioactivity in cells of strain 5-AzCyd-r growing in a medium with glucose (Fig. 3*a*) on the one hand, and the 60% descrease of 5-azacytidine

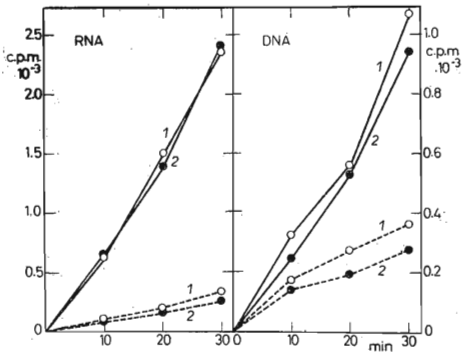

FIG. 5

Effect of Excess Uridine on the Incorporation of Cytidine into Nucleic Acids of *Bacillus subtilis* Sensitive and Resistant to 5-Azacytidine

Incorporation was determined from 1 ml samples of bacterial culture in a minimal Spizizen medium enriched with 1% vitamin-free casamino acids and 0·5% glucose. Turbidity at zero time was A_{575} 0·25. Cytidine-U-[^{3}H] was applied at 0·2 μCi/ml and final concentration 3 μg/ml. Tenfold excess of unlabelled uridine (30 μg/ml) was used.

transport in a minimal medium without glucose (Fig. 3*b*), on the other. This difference suggests that in resistant cells the penetration deficiency is accompanied by another regulating step in the anabolic conversion of the metabolite which may but need not be associated with the mechanism of resistance formation. Such a regulatory step might lie in the naturally occurring difference in substrate affinity of cytidine kinase for cytidine and 5-azacytidine which was observed during phosphorylation of the two compounds *in vitro* by enzyme extracts of mouse liver[24] (the affinity of the kinase for cytidine was about 5 times higher than for 5-azacytidine). Hence the decreased penetration of the antimetabolite may be a sufficient explanation for the formation of resistance. Quantitative changes in enzyme activity (*e. g.* of kinases) are not rare in accompanying resistance[25] and may be significant for the formation of resistance to 5-azacytidine.

The conclusion that the mechanism of resistance is associated specifically with transport and metabolism of 5-azacytidine is borne out by results dealing with the utilization of natural pyrimidine nucleosides which were practically identical for both strains. Similarly results on influence of excess uridine on the incorporation of cytidine do not indicate a different pathway for cytidine conversion in the resistant strain. A definitive scheme depicting the mechanism of resistance of *Bacillus subtilis* cells to 5-azacytidine may be proposed only after studying purified enzyme preparations *in vitro* which is now under way.

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