MECHANISM OF RESISTANCE TO 5-AZACYTIDINE IN *Bacillus subtilis*. III.*
INCORPORATION OF 5-AZACYTIDINE INTO NUCLEIC ACIDS AND ITS EFFECT ON THE TRANSFORMING ACTIVITY OF DNA**

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5-Azacytidine is incorporated into RNA*** (it replaces 28% of the cytosine present) as well as into DNA (52% of the cytosine) of *Bacillus subtilis* cells (SMYW) sensitive to the antimetabolite (5-AzCyd-s). The presence of the antimetabolite in transforming DNA decreases its biological activity, particularly after partial denaturation and at higher pH values. Most affected is the transformation of the adenine marker for which fractions with a relatively higher content of guanine–cytosine pairs in the chain are responsible. In transforming DNA's from strains resistant to the antimetabolite (5-AzCyd-r) no changes are observed that would be caused by the presence of 5-azacytidine in the cultivation medium which corresponds to the inability of these strains to exploit the antimetabolite for the synthesis of their own nucleic acids.

So far it has been possible to demonstrate the incorporation of 5-azacytidine into some bacterial[1,2], plant[3] and animal[4–6] nucleic acids, but with the exception of RNA (ref.[7,8]) it was not possible to follow the effect of incorporation on the biological properties of these natural polymers. In the case of DNA, changes were followed only in the physico-chemical properties and in the structure of preparations isolated from *Escherichia coli* WP 14 growing in the presence of 5-azacytidine[1] where a mutagenic effect[9] of the antimetabolite had been observed before. Fractionation of transforming DNA from *B. subtilis* and correlation of the physico-chemical properties with the transforming activity[10] of the individual fractions for markers from different regions of the genome (ade, leu, met) formed the basis for studying the effect of 5-azacytidine on the biological activity of bacterial DNA. After isolation of strains of *B. subtilis* resistant to 5-azacytidine[11] analogous experiments could demonstrate that we are actually dealing here with the effect of the incorporated antimetabolite.

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*** Abbreviations used: RNA ribonucleic acid, DNA deoxyribonucleic acid, TCA trichloroacetic acid, 5-AzCyd-s and 5-AzCyd-r sensitivity and resistance to 5-azacytidine, respectively.

EXPERIMENTAL

5-*Azacytidine*[12] and 5-azacytidine-[4-^{14}C] (4 mCi/mmol) were prepared in the Department of organic synthesis or in the group of radioactive isotopes of this Institute. $KH_2{}^{32}P_4$ (49 Ci/mg) was from Amersham (England).

Cultures of Bacillus subtilis SMYW 5-AzCyd-s or 5-AzCyd-r, both prototrophic strains[11], grew under aeration at 37°C. The culture for isolation of transforming DNA grew in a normal Spizizen medium[13] enriched with 1% vitamin-free casamino acids (Difco) and with 0·5% glucose, 5-Azacytidine was added to the cultures at a density of A_{575} 0·2 measured in a Kaucky spectrophotometer. Incorporation of 5-azacytidine-[4-^{14}C] during synthesis of nucleic acids *de novo* (double-labelled with ^{32}P and ^{14}C according to ref.[2]) took place in a L.P. medium[14] containing Tris buffer. Recipient strains during transformation were the triple (ade$^-$, leu$^-$, met$^-$) and quadruple (ade$^-$, thr$^-$, leu$^-$, met$^-$) mutants of *B. subtilis*.

Preparations of nucleic acids. Incorporation of 5-azacytidine-[4-^{14}C] into both types of nucleic acids synthesized *de novo* in mutants of *B. subtilis* 5-AzCyd-s and 5-AzCyd-r was followed according to the kinetics of cumulative incorporation of the antimetabolite (shown in detail in Table I) as well as after isolation of DNA and RNA (ref.[2,15]). The other DNA's were obtained by a modified method of Marmur[16] supplemented with one more phenol extraction after treatment with ribonuclease[17]. When studying the effect of DNA purification on the transformation of the individual markers the corresponding preparation were designated as follows: "lysate" — after action of lysozyme (100 μg/ml) on the bacterial suspension the lysate was deproteinized once with a mixture of chloroform–isoamyl alcohol (24 : 1) and precipitated with two volumes of alcohol; after dissolution of the filamentous precipitate the solution was centrifuged for 15 min at 20 000 r.p.m. in the Spinco L50 centrifuge with rotor 50; "RNase-treated" — DNA isolated by modified Marmur's method[17] without the final precipitation with isopropyl alcohol; "final preparation" — DNA isolated by complete method according to Marmur[17]. Preparations purified up to different degrees differed in the content of protein, RNA, heterogeneity of molecular weight and presence of some other impurities[17] (such as polysaccharides, teichoic acid). The final preparations were evaluated on the basis of their spectral characteristics in UV light (A_{max}/A_{min}, A_{260}/A_{280}), hyperchromicity (ΔA) and the T_m values determined in a Unicam SP700 spectrophotometer. Determination of T_m at different pH values of the medium (always in 0·15M-NaCl) served for demonstrating the stability of the isolated preparation[18] of DNA. The sedimentation coefficient $s_{20,w}$ was determined according to sedimentation of the DNA sample (15 μg/ml) in 0·15M-NaCl and 0·015M sodium citrate at pH 7·0 in a Spinco Model E analytical ultracentrifuge.

Transformation experiments were done as described before[19]. The final concentration of transforming DNA during incubation with a competent culture was 1 μg/ml unless otherwise stated. The effect of temperature on the transforming DNA in media of different pH was followed according to the transforming activity of samples heated in an ultrathermostat and quenched in an ice bath at a DNA concentration of 10 μg/ml (heat inactivation was described in Table II). The number of transformants for the individual markers is shown only as the percentage of the number of transformants in the control experiment which was taken as 100% (Table II).

Radioactivity of ^{32}P and ^{14}C was measured in the Packard Tricarb (model 3375) scintillation counter using a toluene scintillation liquid containing 4 g 2,5-diphenyloxazole and 250 mg 1,4-di(2-phenyloxazolyl)benzene in 1 liter toluene.

RESULTS

The replacement of cytosine with the antimetabolite observed in *de novo* synthesized nucleic acids is shown in Table I. Both these values and the curves of cumulative

incorporation demonstrated in Fig. 1 *a*, *b* indicate that the incorporation of the antimetabolite into DNA does not particularly lag behind that into RNA which suggests that 5-azacytidine as diphosphate represents an equally suitable substrate for reducing enzymes as the natural ribonucleoside diphosphates. If a similar experiment was carried out with the resistant bacterial strain (5-AzCyd-r) incorporation could not be demonstrated (Fig. 1 *c*, *d*).

After this finding transformations were performed with DNA preparations isolated up to different degrees of purity (see Experimental) when the transforming activity for ade, leu and met markers was examined. To compare the heat stability the preparations were also examined as to their transforming activity after heating for 30 min to 86°C (in the vicinity of T_m). Table II summarizes the results obtained which indicate that the greatest changes in activity are observed in "lysates", there being considerable differences between native and partially denatured preparations. Using a prolonged incubation of culture in the medium with the antimetabolite and a higher purity of the preparation the decrease in transforming activity all but disappears, apparently due to decomposition of the antimetabolite in the medium during incuba-

TABLE I

Replacement of Cytosine in the Nucleic Acids of *Bacillus subtilis* with 5-Azacytosine

B. subtilis SMYW 5-AzCyd-s culture grew in a L.P. medium[14] under shaking at 37°C (5-azacytidine-[4-^{14}C] 4 μCi/μmol, 0·8 μCi/ml; $KH_2{}^{32}PO_4$ 49 Ci/mg, 5 μCi/ml). At various time intervals, 1 ml samples were transferred into 1 ml 10% TCA, and in parallel into 1 ml 0·6M-KOH. The alkaline solution was incubated at 37°C overnight, acidified with 50% TCA and all the samples were filtered through membrane filters Synpor 6 (pore size 0·40 μm). Radioactivity on filters was estimated in a toluene scintillation liquid. The content of cytosine in DNA was determined chromatographically as 21·5 mol% while in RNA the value of Midgley and McCarthy[26] was taken as 31·4 mol%. The preparations were isolated as described before[2].

Duration of labelling with 5-azacytidine, min	Replaced cytosine, %	
	RNA	DNA
Fraction insoluble in TCA		
10	35·5	43·6
20	24·2	43·0
30	19·6	46·4
45	25·9	48·6
60	23·9	49·0
Isolated preparations		
45	28·1	52·3

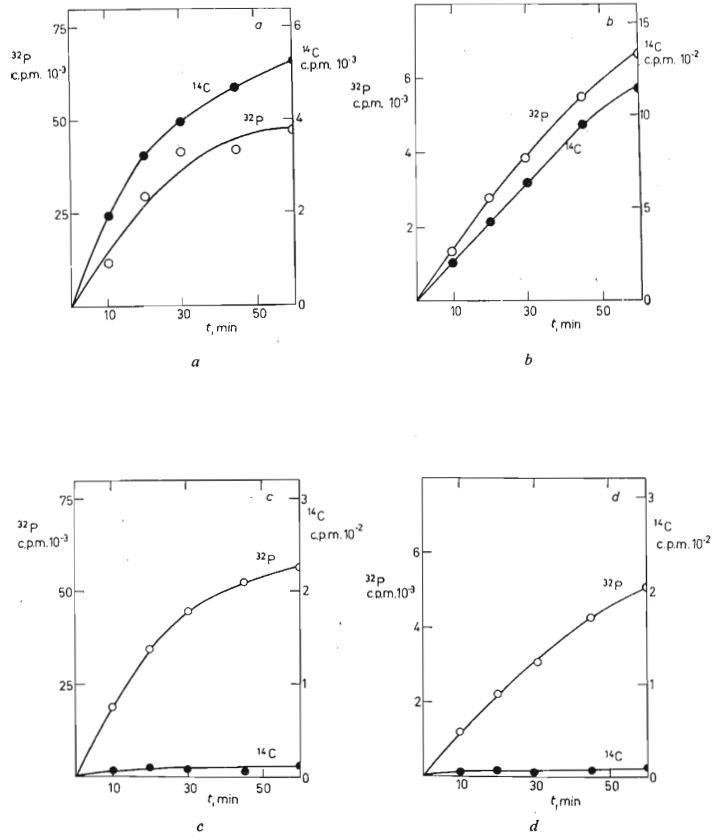

FIG. 1

Synthesis of Nucleic Acids *de novo* and Incorporation of 5-Azacytidine-[4-^{14}C] in Strains of *Bacillus subtilis* SMYW 5-AzCyd-s (A, B) or 5-AzCyd-r (C, D)

a, *c* Incorporation into RNA; *b*, *d* incorporation into DNA; *t* cultivation of culture with radioisotopes in min; c.p.m. radioactivity of newly formed nucleic acid-[^{32}P] and of incorporated 5-azacytidine-[^{14}C].

tion[11,20], and to decrease of heterogeneity of the preparation in the course of purification accompanied by loss of the most labile fragments[1,20], the molecular weights of which were still higher in the original preparation than the critical value decisive for biological activity of fractions. The minimum functional molecular weight of transforming DNA is 10^6 daltons.

The second part of Table II summarizes similar experiments with DNA preparations from a culture of *B. subtilis* 5-AzCyd-r. Since due to the absence of incorporation in resistant cells the minimum effect of the antimetabolite could be expected, a single cultivation interval was selected (30 min), at a concentration of 5-azacytidine

TABLE II

Effect of 5-Azacytidine on the Transforming Activity of DNA in *Bacillus subtilis*

Per cent of transforming activity is referred to the activity of the DNA preparation without incorporated antimetabolite as 100%. The number of transformants in the individual experiments ranged from 0·1 to 0·5% viable cells of the culture tested and was always calculated as an average from three cultures transformed in parallel. The final concentration of transforming DNA was always 1 μg/ml which corresponds to the saturation concentration with the competent culture used. The higher concentration of DNA yielded similar results, the ratios of the individual markers being maintained. Denatured samples of DNA were prepared by heating for 30 min to 86°C and cooled in ice before adding to the competent culture at a concentration of 10 μg/ml.

Concentration of 5-AzCyd μg/ml	Incubation with 5-AzCyd min	Degree of DNA purity	Transforming activity, %					
			ade		leu		met	
			nat.	den.	nat.	den.	nat.	den.
		Bac. subt. SMYW 5-AzCyd-s						
0	0	lysate	100	100	100	100	100	100
25	30	lysate	39	7	59	23	56	32
25	60	lysate	57	11	68	32	66	53
25	120	lysate	61	—	81	—	80	—
0	0	RNase-treated	100	100	100	100	100	100
25	30	RNase-treated	81	50	99	67	96	91
25	60	RNase-treated	78	39	96	94	90	78
0	0	final prep.	100	100	100	100	100	100
25	30	final prep.	74	33	90	39	84	65
25	60	final prep.	91	66	96	75	94	89
		Bac. subt. SMYW 5-AzCyd-r						
0	0	lysate	100	100	100	100	100	100
25	30	lysate	105	98	105	101	112	95
0	0	RNase-treated	100	100	100	100	100	100
25	30	RNase-treated	115	95	107	104	98	99

equal to 25 μg/ml. Similarly, the effect of purification of the DNA preparation was followed only in two steps. A fundamental difference from the sensitive culture is the striking stability and reproducibility of transforming activities for preparations from cultures growing both in the presence and in the absence of the antimetabolite. This agreement holds even for denatured variants. These results then also indirectly show that 5-azacytidine is not utilized by the resistant strain for DNA synthesis and confirm that the differences observed in preparations from sensitive cells are due to incorporation and lability of the unnatural base in the polynucleotide.

Table III shows the basic characteristics of four final highly purified preparations of DNA isolated from the sensitive (DNA 1 and 2) and from the resistant (DNA 3 and 4) strains which grew in the absence (DNA 1 and 3) and in the presence (DNA 2 and 4) of the antimetabolite (50 μg/ml). At first sight, one may observe a difference only in the DNA yield from sensitive cells, cultivated in the presence of the antimetabolite; the part of DNA which can be isolated agrees in its characteristics with the other preparations. The decrease in its T_m value by 1·4°C, although insignificant, might suggest a decreased heat stability of this DNA which would correspond to its readier degradation and would explain also the decreased yield in the process of isolation. The hyperchromicity of all the preparations varies within normal limits which indicates an intact secondary structure.

TABLE III

Characteristics of Preparations of Transforming DNA Isolated from *Bacillus subtilis* Strains Sensitive and Resistant to 5-Azacytidine

A 7-hour culture of the corresponding mutant (5-AzCyd-s or 5-AzCyd-r) was centrifuged, washed and resuspended in a fresh medium without or with 5-azacytidine (50 μg/ml). Cultivation for 45 min (approximately one generation time) under shaking at 37°C. The corresponding DNA was isolated by modified Marmur's method see Experimental.

No	Mutant	5-AzCyd μg/ml	Yield of DNA μg/g (wet bacteria)	A_{max}/A_{min} (A_{260}/A_{280})	$s_{20,w}$ S	T_m °C	Hyperchromicity %
1	5-AzCyd-s	0	1 080	2·27 (1·98)	31·2	85·0	35
2	5-AzCyd-s	50	683	2·32 (1·97)	29·1	83·6	33
3	5-AzCyd-r	0	1 100	2·36 (2·0)	31·4	85·6	36
4	5-AzCyd-r	50	1 069	2·27 (1·80)	28·5	85·5	33

In view of the well-known lability of 5-azacytidine at extreme pH values, the heat stability of DNA was also examined in dependence on the pH of the medium. As may be seen from the curve of dependence of T_m on pH in Fig. 2, the decreased stability of the structure is apparent on comparing DNA 1 and 2 (Table III) only at pH values lower than 4·5 or higher than 9·5 and is reflected in the steeper decline of the curve for DNA 2. In the pH region within the mentioned limits no T_m changes can be observed at all. The effect of extreme pH values corresponds apparently to disturbances in the structure caused by the lability of the antimetabolite which is displayed during the so-called acid and alkaline cleavage due to formation of acyclic decomposition products[20].

Since the transforming activity for the observed markers differed under normal conditions for DNA 1 and 2 only slightly, the transformation was preceded by a partial denaturation of DNA samples at various temperatures (15 h at 50°, 70° and 80°C) and at pH 9·5 and 10·5. With all the markers investigated the transforming activity of DNA 1 increased upon incubation at 50°C and inactivation became apparent only at higher temperatures. At the temperatures used, a change in the sequence of marker sensitivities takes place (Fig. 3, inactivation curves apparently cross each other). A change in the sensitivity at different temperature intervals is reflected even in DNA 2 where, however, no marked increase in activity is observable at 50°C. A striking difference is shown in Fig. 3 when comparing the activity of both DNA's for the adenine marker which, in a culture without 5-azacytidine, is the most resistant

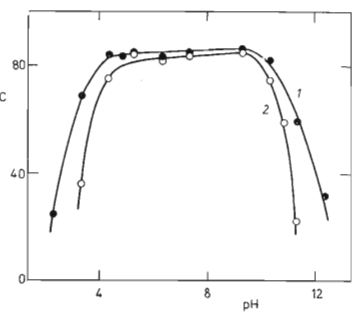

FIG. 2

Dependence of the Melting Temperature (T_m) of Transforming DNA on the pH of the Medium

Transforming DNA isolated from *B. subtilis* SMYW 5-AzCyd-s cultivated without 5-azacytidine (*1*) and in the presence of 5-azacytidine (*2*). The preparations correspond to DNA 1 and 2 of Table III.

one to heat inactivation (up to 75°C) while the DNA 2 preparation isolated from a culture growing in the presence of the antimetabolite transforms the adenine marker under all conditions to the lowest extent. The methionine marker was equally temperature-resistant in both DNA 1 and 2. A similar course of heat inactivation may be observed also at pH 10·5 while at pH between 4·5 and 9·0 the differences are insignificant. If preparations of DNA 3 and 4 isolated from resistant cultures were used as transforming factors no differences were observed in their biological activity and here, too, the adenine marker corresponded by its sensitivity to the DNA 1 preparation (Table III).

DISCUSSION

It appears that the antimetabolite is incorporated by the sensitive strain into RNA as well as DNA. Since the radioactive preparation of the antimetabolite is labelled in position $C_{(4)}$ one can observe incorporation even in cases that the unstable triazine cycle is split in the macromolecule the nucleoside bond to the sugar residue being preserved; this is met even under conditions of alkaline hydrolysis of RNA when incorporation into DNA is followed. In this way it cannot be decided, however, whether an unchanged 5-azacytosine heterocycle is incorporated since *in vitro* the strain used displayed a cytidine deaminase activity[21]. It is thus not impossible that 5-azauridine, even if it cannot be prepared in the normal nucleoside form in the

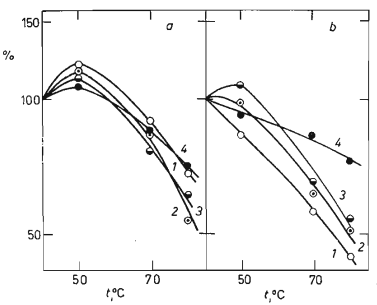

FIG. 3

Transforming Activity (in %) of Partially Inactivated DNA Isolated from *Bacillus subtilis* SMYW 5-AzCyd-s

a Transforming DNA without 5-azacytosine; *b* DNA containing 5-azacytosine; *t* denaturation temperature to which the samples of DNA were exposed for 15 h at pH 9·5; % transforming activity of DNA for ade (*1*), thr (*2*), leu (*3*), and met (*4*) markers.

laboratory[12], preserves its nucleoside structure after formation from 5-azacytidine in the cell so that it can be incorporated into nucleic acids, particularly into RNA. So far, incorporation of 5-azauracil could not be demonstrated. It is also contradicted by the negligible antibacterial efficiency of 5-azauridine[22] (at 10^{-3}M) as compared with 5-azacytidine (at $10^{-7}-10^{-6}$M).

Whether the antimetabolite is incorporated into DNA unchanged or in a different form, it may be always assumed that the labile triazine cycle may cause disturbances in the structure of nucleic acids and hence bring about their functional inactivation. In the case of DNA this is supported by the greater lability of its structure after incorporation of the antimetabolite and by change in transforming activity, reflected first of all after a denaturation treatment and after an increase of the pH of the medium, most pronouncedly during transformation of the ade marker. The fact that we are dealing here with a true effect of the antimetabolite is supported by the negative results obtained under identical conditions with *B. subtilis* 5-AzCyd-r where no antimetabolite incorporation or other changes and differences observed in sensitive cells could be demonstrated.

According to our previous results with the fractionation of transforming DNA from *B. subtilis* SMYW the ade marker behaves as a part of the polydeoxyribonucleotide chain with a relatively higher content of guanine-cytosine pairs[10]. In spite of the fact that a certain gradient in the guanine–cytosine content along the whole genome of the bacterial cell could be observed the differences were most striking between the ade and met markers which are farthest apart on the linear genetic map[23]. The preferential influence on the ade marker in transforming DNA is thus explainable by the increased content of the antimetabolite in the corresponding part of DNA and hence by a readier disturbance of the corresponding structure. This is marked particularly during inactivation at lower temperatures (50° and 70°C) where practically no inactivation is observed in a normal DNA preparation. An explanation on the basis of an increased GC content is in agreement with chemical treatment of transforming DNA with hydroxylamine when the adenine marker is most sensitive[24].

Comparison of the properties of the final, highly purified preparations of DNA isolated from cultures growing in the presence or in the absence of 5-azacytidine confirms that under normal circumstances the 5-azacytosine ring can fully replace natural cytosine, *i.e.* even fulfil its normal structure-stabilizing function[25] (*e.g.* take part in the formation of hydrogen bonds in the polynucleotide). On the other hand, however, the greater tendency to structure disturbances observed during isolation and after treatment with extreme temperatures and pH values indicates that the lability of such a structure would be reflected in the cell first of all in replication and transcription.

The properties of DNA, especially the change in molecular weight and T_m, were affected much more substantially in *E. coli* WP 14 (ref.[1]) than in *B. subtilis* even

if the extent of replacement of cytosine with 5-azacytosine during incorporation is practically identical[2]. This difference might be accounted for by a more even incorporation of the antimetabolite along the DNA chain in *E. coli* so that structural changes would affect a greater part of the genome, in contrast with *B. subtilis* (where first of all the ade marker is affected). However, an identical fractionation of DNA from *E. coli* where the content of guanine and cytosine would be evaluated, has not been carried out and hence the above explanation remains uncorfirmed.

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