Incorporation of Thymine into Prototrophic and Thymine-dependent Mutants of *Bacillus anthracis*

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SUMMARY

Mutants of *Bacillus anthracis* which were prototrophic in a defined medium below 32° required either thymidine (mutant *thy*) or low concentration of thymine (mutant *thylr*) for growth at 37°. No thymidylate synthetase was produced by these mutants at 37°. Normal enzyme activity was found in bacteria grown at 25°. Absence of thymidylate synthetase activity in bacteria grown at 37° could not be accounted for either by a heat-sensitive enzyme protein or by an inhibitor of this enzyme activity.

The incorporation of thymine by prototrophic bacteria of *Bacillus anthracis* was negligible at either temperature. Mutant *thylr* and its prototrophic revertant both readily took up thymine at 37° but not at 25°. Thymine uptake by both prototrophic and *thy* bacteria was markedly enhanced by the presence of any of the deoxyribonucleosides except thymidine. Moreover, thymidine antagonized thymine uptake. No thymidine phosphorylase activity was detected in *B. anthracis*, but phosphorolytic decomposition of deoxyuridine was found and that of deoxyadenosine seemed likely. The phosphorolytic reaction of deoxyuridine resulted in an accumulation of deoxyribose by *thylr* bacteria, whilst both wild-type and *thy* bacteria catabolized this sugar. The secondary mutation in *thy* bacteria presumably resulted in a defect in synthesis of 1,5-phosphodeoxyribo mutase or deoxyribose 5-phosphate aldolase (E.C. 4.1.2.4). Although no thymidine phosphorylase was detected in *B. anthracis*, resting suspensions of this bacterium transferred the deoxyribosyl moiety from thymidine to thymine. This indicates the presence of the enzyme nucleoside purine (pyrimidine) deoxyribosyl transferase in *B. anthracis*. This particular enzyme may also be involved in the increased thymine uptake in the presence of different deoxyribonucleosides.

INTRODUCTION

The isolation of mutants from *Bacillus anthracis* unable to synthesize thymidine nucleotide has been reported (Ivánovics, 1964). These mutants required thymine for growth only when they were cultivated in a defined medium for *B. anthracis* at temperatures above 32°. To approach the uniform nomenclature in bacterial genetics recommended by Demerec *et al.* (1966), mutant VC-TdR⁻ isolated from prototrophic bacteria will be referred to as strain *thy*; one of its variants, a secondary mutant, isolate VC-T⁻, as strain *thylr* (thymine low requirement). Strain *thy*, as noted previously, had about a 50-fold greater thymine requirement than did *thylr*. Strain *thy* grew normally in the presence of thymidine (TdR), but this nucleoside inhibited the multiplication of *thylr* bacteria (Ivánovics, 1964). The mutants grown in the absence of pyrimidines at low temperature showed a normal replication of macromolecules and normal cell morphology. A shift up of the cultivation temperature to

37° in the absence of thymine resulted in an unbalanced synthesis of macromolecules followed by the death of both *thy* and *thylr* bacteria (Ivánovics, 1963, 1964). Our earlier study of the thymine auxotrophs was restricted to nutritional experiments. The purpose of the present work was to elucidate the site and nature of the block in the thymine pathway, and to gain some insight into thymine incorporation by tracer experiments and enzymic studies.

METHODS

Bacteria. The original mutants of *Bacillus anthracis* produced an extracellular proteolytic enzyme during growth. Since a contamination of cell extracts with proteolytic enzyme would affect the enzymic activity under investigation, non-proteolytic mutants of *thy* and *thylr* were isolated. Bacteria of these strains were treated with ethylmethanesulphonate and the surviving organisms cultivated until marked sporulation occurred. Spores thus obtained were spread on milk agar plates and incubated. Colonies which developed without a proteolytic halo were purified by streaking. Stable non-proteolytic derivatives of strains *thy* and *thylr* were used in all experiments. A prototrophic revertant obtained from *thylr*, referred to as strain *thylrrev*, and a non-proteolytic wild-type strain NPA which came from the collection of Professor H. Smith (Birmingham) were also used in these studies. A non-capsulogenic derivative of NPA which will be referred to as strain P, was used throughout. Spores were prepared as described previously (Ivánovics, 1962), and a single batch of spores from each strain was used to provide inocula.

The strain *Escherichia coli* B was from the collection of this laboratory.

Media. A basal casein hydrolysate medium (BCM) was used for cultivating bacteria under defined conditions; it contained 1 µg. thiamine/ml. and was prepared as previously described (Ivánovics, Varga & Marjai, 1963). Milk agar plates were made from yeast-extract peptone agar by adding 40% (v/v) centrifuged milk.

Chemicals. DL-L-tetrahydrofolic acid was prepared as recommended by Kisliuk (1957). All other chemicals used were reagent grade purchased from different commercial firms.

TPB buffer. 0.05 M-tris + HCl buffer (pH 7.2) containing 0.1 mM-sodium phosphate.

Extinctions. The extinctions of the bacterial suspension was measured with a Spectromom 20, at 600 mµ. A spectrophotometer, MOM 201 (Budapest), served to measure extinction at different wavelengths.

Growth of organisms for enzyme assays. Bacteria of strains *thy* and *thylr* were grown in BCM either in the absence of pyrimidine at 24° or in the presence of thymidine and thymine, each at 5 to 10 µg./ml. at 37°. Except where otherwise stated, no pyrimidine was present in the medium when strains P, *thylrrev* or *E. coli* were grown.

To obtain a washed suspension, a culture in the exponential phase was centrifuged, the deposit washed with TPB, and suspended in TPB to give an extinction value of 0.7; this corresponded to 0.6 mg. dry bacteria/ml.

Crude bacterial extracts were made from 200 ml. cultures harvested in the middle log. phase as follows. The culture was rapidly cooled, centrifuged and the deposit washed with TPB. The packed bacteria were taken up in 5 ml. TPB and treated in a MSE Ultrasonic Power unit at 2° to 4° for 4 min. The homogenate was sharply centrifuged, its supernatant fluid removed and 50 mg./streptomycin sulphate added to it; the bulky precipitate caused by the streptomycin was removed by centrifugation.

Assay for thymidylate synthetase activity. A fraction, obtained from the crude bacterial extracts by precipitation with between 26% and 35% (w/v) ammonium sulphate, as recommended by Wahba & Friedkin (1962), was assayed for its activity by the Method III of these authors. The assay system was incubated at 25° for 60 min., and enzymic activity calculated from the values measured between 2 and 12 min. One enzyme unit is defined as the amount of enzyme catalysing the formation of 1 μ mole thymidylate/hr/mg. protein.

Thymidine:orthophosphate deoxyribosyl transferase. (Thymidine phosphorylase, E.C. 2.4.2.4.) Three tests were used to demonstrate this enzyme activity of bacteria. (1) The crude bacterial extract was mixed with an equal volume of 0.04 M-cysteine hydrochloride solution and 0.1 M-potassium phosphate buffer (pH 7.2) and the activity measured by the method of Razzel & Khorana (1958). (2) A washed bacterial suspension containing 0.6 mg. dry bacteria/ml. was treated with toluene and assayed as recommended by Fangman & Novick (1966). (3) Either strain P or *thy* was grown in BCM containing 0.1 to 1.0 mM of thymidine at 37° overnight; a 1/20 dilution was made from this inoculum with the same medium and incubated until the extinction reached to 0.7 to 0.8. The supernatant fluid of the culture was sucked through a membrane filter (no. 4 Göttigen), the filtrate mixed with the same volume of 0.3 N-NaOH, and its extinction measured at 300 m μ . The conversion of thymidine to thymine was calculated from the increase of extinction as recommended by Hotchkiss (1948).

Tests for enzymic degradation of uridine deoxyriboside (UdR) and adenine deoxyriboside (AdR). To assay the enzyme capable of catalysing the phosphorolysis of UdR washed bacteria were made up in TPB with 1 mM-UdR. Assay systems were prepared in duplicate, with and without 1 mM sodium arsenate, both were incubated for 60 min. at 37°. When 1 μ mole UdR is converted to uracil, there is an increase of 4.1 in extinction value at 290 m μ in the presence of 0.1 N-NaOH. This served to estimate the enzyme activity from the amount of uracil formed. When the enzyme activity was measured by the formation of sugar from the nucleoside, a sample from the assay system was deproteinized with HClO₄ (0.5 M), and the clear supernatant fluid heated in boiling water for 3 min. The amount of deoxyribose, both free and phosphorylated, was determined by the diphenylamine test (Burton, 1956). A sample was also taken from the assay system without arsenate, deproteinized by Ba(OH)₂ + ZnSO₄ (Manson & Lampen, 1951) to determine the free sugar.

The degradation of AdR could not be followed in a way similar to that for UdR. Therefore, a qualitative test was devised involving thin-layer chromatography; this gave an indication of the decomposition of AdR by washed bacteria. The technique of chromatography was as described in the next paragraph.

Assay for nucleoside:purine (pyrimidine) deoxyribosyl-transferase (E.C. 2.4.2.6.). Different unlabelled deoxyribonucleosides were used as donors and tritiated thymine as acceptor for the deoxyribose moiety. The reagent to detect the enzyme activity contained 8 m-mole of the deoxynucleoside and 3 m-mole of tritiated thymine (2 μ C/ml. activity) dissolved in TPB diluted 1/10 with water. Two ml. of the reagent was mixed with same volume of either washed bacterial suspension or the uncentrifuged ultrasonic fraction and incubated for 60 min. at 37°. The assay system was rapidly cooled and centrifuged; 12 mg. charcoal were added to 3 ml. of the supernatant fluid, and shaken for 15 min. The charcoal was sedimented, and eluted by a mixture of 8 vol. ethanol + 1 vol. water + 1 vol. ammonium hydroxide solution (0.88 sp.gr).

Elution done 5 times with 5 ml. portions of the mixture. Recovery of the radioactivity in the pooled eluate amounted to 95 %. With the aid of an intensive cool air stream, the volume of the pooled eluate was evaporated to about 1 ml. The exact volume if the concentrate was measured by weight on an analytical balance. Samples (20 to 40 μ l.) of eluate were put on a thin layer of cellulose (MN 300) containing 3 % of $\text{Al}(\text{OH})_3$, and run with a solvent of 15 ml. *n*-butanol + 60 ml. acetone + 20 ml. water + 5 ml. concentrated ammonium hydroxide. A very good resolution of thymine and thymidine was achieved in this way. R_f values: thymine = 0.55; thymidine = 0.66; UdR = 0.43; uracil = 0.32.

Material scraped off the spots of thymine and thymidine was suspended in scintillation liquid and its activity counted. Scintillation liquid was 0.5 g. POPOP and 5 g. PPO in 1 l. toluene. Radioactivity was counted in a TRI Carb Packard, Model 2101, scintillation spectrometer. The amount of radioactive thymine converted to thymidine was calculated from the percentage distribution of the label between the base and its nucleoside. Neglecting the loss of activity which occurred during isolation of products by charcoal treatment, enzyme activity was expressed by the amount of thymidine formed μ mole/hr/ml. bacterial suspension.

Determination of bacterial multiplication. *Bacillus anthracis* is a chain former, therefore the bacterial protein content of the culture was determined instead of colony count. A 0.5 ml. sample was mixed with 0.5 ml. cold 10 % (w/v) TCA and centrifuged; the bacteria were washed twice with 5 % TCA, finally suspended in 0.5 ml. of 10 % NaOH, and kept overnight in cold. The protein content of the extract was determined by the method of Lowry *et al.* (1951). The number of generations was calculated by the formula $(\log A_t - \log A_0)$ divided by 0.301. A is the amount of protein per ml. at 0 and t times, respectively.

Incorporation of thymine into bacteria. Tritiated thymine ($-\text{CH}_3\text{-}^3\text{H}$) was obtained from the Hungarian State Isotopic Laboratory. The stock solution contained 1.173 mg. thymine/ml. with an activity of 1.077 mC/ml. Labelled thymine was diluted with cold thymine as appropriate.

Tritiated thymine, 5 μ g./ml. (1 μ C/ml. activity), was added to bacteria growing exponentially in BCM. Samples of the bacterial culture (0.1 ml.) were taken at intervals and added to 5 ml. cold 5 % TCA containing 10 μ g. thymine/ml., sucked through a membrane filter, followed by several washings of the filter with 2 ml. cold TCA and cold saline. Radioactivity of the dried filter was measured; counts given by the zero time sample was considered background activity and subtracted from the timed sample.

RESULTS

Heat-activated block in the de novo pathway of the chromosome replication

Previous studies (Ivánovics, 1964) suggested the existence of a block in thymidylate-5-phosphate synthesis which exerts its effect on the chromosome replication in the mutants only at an elevated temperature. Table I shows that no thymidylate synthetase activity was detected in preparations obtained from mutants grown at 37° with exogenous pyrimidine. However, this enzyme activity was found in preparations from the mutants cultivated at 25° and in the preparations obtained from prototrophic wild-type and revertant bacteria (strain *thytlrrev*) grown at 37°. The same enzyme activity was recovered from *Escherichia coli* cultivated at 37°.

Table 2 shows that there was no difference in the heat sensitivity of the enzymic activity of preparations obtained either from prototrophic or from thymine-dependent bacteria.

The lack of thymidylate synthetase activity in preparations of *thyltr* bacteria cultivated with exogenous thymine at 37° was not due to the presence of an inhibitor of this enzyme since, if this were the case, the inactive preparation of *thyltr* would inhibit the enzyme activity of a preparation from the prototrophic bacteria. No such an effect was observed.

Table 1. *Specific thymidylate synthetase activity of individual preparation obtained from bacteria grown at low and elevated temperature*

For growing strains *thy* and *thyltr* at 37°, casein hydrolysate medium BCM contained 5 µg./ml. thymidine and thymine, respectively. Zero value indicates that less than 0.01 enzyme unit was found in each of 2 to 4 individual preparations investigated

Bacterium	Specific enzyme activity: µmole/ hr/mg. protein from bacteria grown at	
	25°	37°
<i>B. anthracis</i> , P	N.I.*	0.078
<i>B. anthracis</i> , <i>thy</i>	0.065	0
	0.078	.
<i>B. anthracis</i> , <i>thyltr</i>	0.075	0
	0.077	.
	0.081	.
<i>B. anthracis</i> , <i>thyltr</i> rev	N.I.	0.088
<i>E. coli</i> B	N.I.	0.079

* Not investigated.

Table 2. *Inactivation of thymidylate synthetase activity of preparations by heating at 50° in tris buffer-Na phosphate at pH 7.2*

Time of heating (min.)	Specific enzyme activity of preparation from strain	
	<i>thyltr</i>	P
0	0.075	0.070
5	0.070	0.065
10	0.052	0.047
15	0.032	0.032
20	0.023	0.019
30	0.009	0.013

Incorporation of thymine by strains of Bacillus anthracis

A summary of results is shown in Table 3. Only an insignificant incorporation of label was detected in prototrophic bacteria at either temperature and in the mutants at 25°. On the other hand, a marked uptake of thymine by strains *thyltr* and *thyltr* rev was observed at 37°, although the revertant was a thymine-independent derivative of *thyltr* with thymidylate synthetase activity at 37°. A striking feature of the thymine

incorporation was its dependence on temperature. Apparently the intactness of the pathway leading to thymidylate synthetase was not the only factor which regulated thymine uptake. Strain *thy* could not be examined under identical condition since this strain does not grow at such a low concentration of thymine.

Fig. 1

Fig. 2

Fig. 1. The effect of different deoxyribonucleosides on the incorporation of tritiated thymine into prototrophic strain P of *Bacillus anthracis*. A culture growing exponentially in casein hydrolysate medium BCM containing 5 μ g. labelled thymine/ml. with 1 μ C activity/ml. was divided into four samples at 20 min.; they were supplemented with different nucleosides; 20 μ g./ml. each. ●, Thymine only (T); ○, T + AdR; △, T + UdR; ▲, T + CdR; □, bacterial protein in culture (mg./ml.). The growth rates of the bacteria were identical in all cases; the generation time was 45 min.

Fig. 2. The incorporation of tritiated thymine into prototrophic *Bacillus anthracis* in the presence of AdR and TdR. 20 μ g. AdR/ml. and 5 μ g. tritiated thymine/ml. with 1 μ C activity/ml. was added to an exponentially growing culture. The culture was divided in two parts at 20 min.; one of them was reincubated while the other was supplemented with 5 μ g. thymidine/ml. before reincubation. Incorporation of thymine in systems: ○, T + AdR; ●, T + AdR + TdR; □, weight of bacterial protein.

Table 3. Thymine incorporation into *Bacillus anthracis* at 25° and 37°

Bacteria were grown overnight at 25° in casein hydrolysate medium BCM containing unlabelled thymine 5 μ g./ml. The cultures were diluted 1/10 with the same medium and reincubated at 37° until their extinction reached to 0.2; this was considered 0 time when tritiated thymine 1 μ C/ml. was added to the cultures. Reincubation was either 25° or 37°.

Time of cultivation (min.)	Strain P		Strain <i>thy</i>		Strain <i>thyltr</i>		Strain <i>thyltrrev</i>	
	25°	37°	25°	37°	25°	37°	25°	37°
	C.p.m./mg. bacterial protein							
25	350	351	350	N.I.*	290	3970	351	3840
75	480	490	720	N.I.	485	8542	515	10344

* Not investigated.

Exogenous thymine utilization by strains of *Bacillus anthracis* was increased in the presence of various deoxyribonucleosides. Figure 1 shows that prototrophic bacteria readily incorporated thymine in the presence of deoxyribonucleosides; AdR was most effective in this respect, UdR is next, whilst CdR exhibited only a moderate effect. By contrast, adenosine, deoxyribose and thymidine were unable to enhance the effect of label-incorporation into the acid-insoluble material of prototrophic bacteria. Moreover, thymidine inhibited thymine uptake into prototrophic bacteria even in the presence of AdR (Fig. 2).

The addition of thymidine to the culture stopped thymine uptake by prototrophic bacteria, although this did not affect the growth rate of the bacteria. This particular effect of thymidine was also valid for *thy* and *thytlr* bacteria when they were grown at 37°. Multiplication of *thy* bacteria was not supported by a low concentration of thymine (5 µg./ml.), therefore no label was taken up under this condition. Thymidine itself, on the other hand, gave a good growth of this organism. Deoxyribonucleosides (e.g. AdR) augmented the thymine uptake by *thy* bacteria, resulting in label incorporation and growth. Label incorporation, however, was blocked when thymidine was present in the culture (see Fig. 3).

Fig. 3

Fig. 3. The effect of different deoxyribonucleosides on the incorporation of tritiated thymine into *Bacillus anthracis thy*. Bacteria were grown in casein hydrolysate medium BCM containing 5 µg. thymidine/ml. until an extinction value of 0.3. The bacteria were centrifuged down, washed with BCM medium and suspended in 40 ml. BCM medium which contained 5 µg. thymine/ml. with 1 µCi activity/ml. The suspension was distributed in 10 ml. samples and the samples supplemented with nucleosides. The growths of individual cultures were identical. □, Protein content of the cultures; generation time of bacteria, 60 min. Incorporation of thymine: ▲, 20 µg. AdR/ml. + thymine; ○, 5 µg. thymidine/ml. + thymine; △, AdR + thymidine + thymine.

Fig. 4

Fig. 4. The growth of strain *thytlr* of *Bacillus anthracis* and the incorporation of thymine in the presence and absence of thymidine. Bacteria were grown in casein hydrolysate medium BCM with 5 µg. unlabelled thymine/ml.; 1 µCi tritiated thymine/ml. was added at 0 time and the culture reincubated. The culture was divided into equal volumes; at 20 min. thymidine 1 µg./ml. was added to one of the samples; the other served as control. Incorporation of thymine: ●, in absence of thymidine; ▲, in presence of thymidine; ○, bacterial protein without thymidine, and, △, with thymidine.

The inhibitory effect of thymidine on thymine uptake by *Bacillus anthracis* was most notably exhibited by mutant *thytlr*; addition of thymidine to the culture growing with thymine at 37° stopped both label-incorporation and growth (Fig. 4).

Examination of enzymes involved in metabolism of pyrimidine nucleosides

Deoxynucleoside phosphorylases. Phosphorolytic reactions are known to participate both in the degradation of nucleosides in the presence of orthophosphate yielding deoxyribose 1-phosphate and base, and in the formation of deoxyribonucleoside from sugar-phosphate with any of the nucleic acid bases. As far as we are aware, nothing is known of these enzymic reactions in *Bacillus anthracis*; therefore it appeared rewarding to extend our study in this direction. Crude extracts from bacteria grown in the presence of 10 to 20 µg. thymidine/ml. were assayed for thymidine phosphorylase

activity. Material from mutant strain *thytlr* could, of course, only be obtained from bacteria cultivated without this nucleoside. Values were found to be very low in all cases; specific enzyme activities of preparations varied between 0.2 and 0.3, whilst those from *Escherichia coli*, for comparison, showed a value of 5.2 to 5.8. When enzyme assays were made with washed bacterial suspensions, no thymidine phosphorylase activity was detected in the strains of *B. anthracis*. On the other hand, the *E. coli* suspension was effective in degrading thymidine by phosphorolysis.

Growth of mutant *thy* was not sustained by a low concentration of thymine, although it grew well on thymidine concentrations as low as 2 to 5 $\mu\text{g./ml.}$ Should this organism

Table 4. *Degradation of thymidine into thymine by exponentially growing bacteria*

Flasks with casein hydrolysate medium BCM containing either 0.1 or 1.0 μM of thymidine were inoculated and incubated overnight; dilutions of cultures were made with same medium and reincubated at 37° with shaking. The cultures were harvested at an extinction of 0.7 in Expt. 1, and at 0.5 in Expt. 2; they were membrane-filtered and mixed with an equal volume of 0.2 N-NaOH. Their extinctions were measured at 300 m μ ; a non-inoculated medium served as a blank.

Bacterium	TdR concentration in medium (μM)			
	Expt. 1:0.1		Expt. 2:1.0	
	Change of extinction*	Thymine formed ($\mu\text{mole/ml.}$)	Change of extinction	Thymine formed ($\mu\text{mole/ml.}$)
<i>B. anthracis</i> , P	+0.008	0.004	+0.006	0.003
<i>B. anthracis</i> , <i>thy</i>	-0.040	Zero value	-0.003	Zero value
<i>E. coli</i> , B	+0.192	0.106	+0.260	0.144

* Multiplied by 2 when the amount of thymine was calculated. Minus values may derive from an exogenous product released by bacteria during growth.

have an effective thymidine phosphorylase activity, the bacteria would be expected soon to exhaust the low concentration of thymidine in the medium by splitting it to thymine which would not be utilizable. To test this, *Bacillus anthracis* and *Escherichia coli* were cultivated in the presence of thymidine and the conversion of thymidine to thymine determined. Thymidine phosphorylase is known to be an inducible enzyme in *E. coli*, therefore bacteria used as the inoculum were also grown in the presence of high concentrations of thymidine. Table 4 shows the results. *Escherichia coli* split thymidine considerably during its growth, whereas the strains of *B. anthracis* did not exhibit a significant degradation of it. The very low rate of thymidine degradation either with crude extracts of *B. anthracis* or with living bacteria did not furnish evidence for the existence of thymidine phosphorylase in this organism.

In contrast to these observations, a marked phosphorolysis of UdR was shown by *Bacillus anthracis*. As shown by Table 5, a resting bacterial suspension of prototrophic *B. anthracis* decomposed UdR, releasing free base but no sugar; however, both base and sugar accumulated in equimolar quantity in the assay system with arsenate. Mutant *thytlr* cleaved UdR by releasing both base and sugar in the absence of arsenate, which indicates that deoxyribose was not catabolised by this mutant. An evidence for phosphorolytic cleavage of UdR was furnished by the identification of sugar-phosphate in the assay system of *thytlr* bacteria when a sample was taken from

it, treated with Zn + Ba reagent (Manson & Lampen, 1951), and the sugar determined in the supernatant fluid. In Expt. 1 (see Table 5) only 0.04 μ mole diphenylamine-positive substance/hr./ml. was left in the supernatant fluid, the bulk of deoxyribose was removed by the Zn + Ba treatment which indicated its phosphorylated form.

Table 5. Release of uracil and deoxyribose from UdR by resting bacterial suspensions of *Bacillus anthracis*

Prototrophic bacteria were grown in the presence of UdR 10 μ g./ml.; the concentration of UdR in assay system was 1 μ M, incubation at 37° for 30 min. The growth of strain *thyltr* was sustained by thymine 5 μ g./ml.

<i>B. anthracis</i> strain	Expt. no.	Presence of arsenate	Products in suspension (μ mole/hr./ml.)	
			Free uracil	Deoxyribose
Prototrophic	1	—	0.28	0.02
	2	—	0.29	0.02
	2	+	0.28	0.28
<i>thyltr</i>	1	—	0.31	0.28
	2	—	0.25	0.21

Table 6. Deoxyribosyl transfer by resting bacteria in the presence of various deoxyribonucleosides as donors

Assay system contained tritiated thymine 3 μ mole/ml., various deoxyribonucleosides as donors, and the washed bacterial suspension. More than one experiment was made with each assay system; the results show the mean values.

Donor	Amount of radioactive thymidine formed by washed bacterial suspension of:			
	<i>B. anthracis</i> strain:			
	<i>E. coli</i>	P	<i>thy</i>	<i>thyltr</i>
	Thymidine formed (μ mole/hr./ml.)			
UdR	1.73	1.09	1.23	1.36
TdR	0.78	0.50	0.81	0.64
CdR	1.23	1.05	1.14	1.41
AdR	<0.03	<0.03	<0.03	<0.03
GdR	<0.03	<0.03	<0.03	<0.03

The degradation of AdR by the prototrophic and mutant strains of *Bacillus anthracis* was also demonstrated. The presence of both adenine and hypoxanthine in the assay system indicated the cleavage of AdR and partial deamination of the base. No attempt was, however, made to show the presence of phosphate-sugar in the reaction mixture. The phosphorolysis of AdR by *B. anthracis* was therefore only presumable.

Nucleoside:purine (pyrimidine) deoxyribosyl transferase. In this enzymic reaction tritiated thymine served as an acceptor of the deoxyribose moiety which came from different purine or pyrimidine deoxyribonucleosides. The results are summarized in Table 6. In some experiments, bacterial suspensions were homogenized by ultrasonic treatment and used as an enzyme preparation in the presence of UdR as donor. None of the homogenates of the strains of *B. anthracis* was capable of catalysing the transfer reaction, in contrast to the test made with the *Escherichia coli* homogenate

where tritiated thymidine was formed; however, it was somewhat less ($1.47 \mu\text{mole/hr/ml.}$) than that formed by intact bacteria. In summary, an enzyme activity catalysing the transfer of the deoxyribosyl radical from pyrimidine deoxyribosides by the whole organisms of *B. anthracis* was found; however, neither AdR nor GdR were capable of taking part in this reaction.

DISCUSSION

With different species of Enterobacteriaceae, variable phenotypes of thymine mutants, depending on the quantitative requirements of thymine, have been described (Alikhanian *et al.* 1966; Brietman & Bradford, 1967*a*; Roepke, 1967). It has been considered that thymine mutants with low base requirements are actually double mutants (Harrison, 1965; Stacey & Simpson, 1965; Alikhanian *et al.* 1966; Breitman & Bradford, 1967*b*; Eisenstark, Eisenstark & Cunningham, 1968), just as it was concluded in the case of thymine-dependent mutants of *Bacillus anthracis* (Ivánovics, 1964). It has been pointed out by Breitman & Bradford (1967*b*) that the second mutation results in lack of deoxyribose aldolase activity which is associated with a low thymine requirement of the bacteria. The ability to incorporate thymine into DNA at a low exogenous concentration also may be caused by a mutation involving the loss of deoxyribomutase (Munch-Petersen, 1968; Barth, Beacham, Ahmad & Pritchard, 1967). The consequence of the second mutation is an intracellular accumulation of deoxyribose-1-phosphate which increases the utilization of exogenous thymine (Kammen, 1967; Breitman & Bradford, 1967*b*). It has been suggested that genes controlling the catabolism of deoxyribonucleosides constitute an operon (Barth *et al.* 1968). This operon controls the production of thymidine phosphorylase, purine nucleoside phosphorylase (E.C. 2.4.2.1), deoxyribomutase and deoxyriboaldolase (E.C. 4.1.2.4) which are capable of catalysing either the biosynthesis or breakdown of deoxyribonucleosides. The operon conception concerning deoxyribose catabolism has been advanced and the *deo* designation for this operon was suggested to indicate the genes determining enzymes necessary for growth on deoxythymidine or deoxyuridine as sole carbon source (Lomax & Greenberg, 1968). Any of the mutations leading to a low thymine requirement in strains lacking thymidylate synthetase are also associated with a phenotype characterized by a sensitivity of bacterial growth to thymidine or to other deoxyribonucleosides (Alikhanian *et al.* 1966; Eisenstark *et al.* 1968). As shown by Barth *et al.* (1967), thymine mutants of *Salmonella typhimurium* highly sensitive to thymidine are lacking in deoxyriboaldolase, and the less sensitive mutants lack deoxyribomutase.

In the present work the first mutation in *Bacillus anthracis* affected the capacity of bacteria to produce thymidylate synthetase at 37° . Similarly, this enzyme was detected in the extracts of *thytlr B. anthracis thytlr* bacteria grown with exogenous thymine at 37° ; however, effective enzyme activity was found in extracts obtained from these mutants when grown at 25° . Apparently the heat sensitivity of DNA replication in the mutants could not be accounted for either by an alteration in the structure of enzyme protein which renders the enzyme extremely heat labile, or by an inhibitor of the thymidylate synthetase activity. It seems that the synthesis of this enzyme itself is a heat-labile process. Heat-sensitive thymine mutants are not uncommon among bacteria. Alikhanian *et al.* (1966) found that half their thymine mutants isolated from *Escherichia coli* K 12 mapping at the site of *thy* locus were temperature-

sensitive. We would stress that the majority of thymine auxotrophs of *B. anthracis* isolated by using ethylmethanesulphonate as mutagen, needed thymidine only at temperatures higher than 32° (Ivánovics *et al.* 1963), like the strain *thy* used in this study.

With the exception of thymidine, all the deoxyribonucleosides tested enhanced the incorporation of tritiated thymine into acid-insoluble material of *Bacillus anthracis*. Moreover, thymidine totally blocked thymine utilization by prototrophic bacteria and thymine auxotrophs. The rate of bacterial multiplication, however, appeared to be independent of exogenous thymine uptake except when thymidine was added to cultures of mutant *thytlr* growing in the presence of thymine; in this case both thymine incorporation and growth were arrested.

A striking feature of the enzymic make-up of *Bacillus anthracis* is the lack of thymidine phosphorylase activity. On the other hand, definite evidence was found that UdR was decomposed by phosphorolysis by prototrophic and by *thytlr* bacteria. The absence of deoxyribose from the assay system of prototrophic bacteria indicated that deoxyribose-1-phosphate formed by the phosphorolytic enzyme was metabolized. Mutant *thytlr* was found, in contrast, to be incapable of metabolizing the sugar-phosphate formed by the phosphorolysis of UdR, indicating that either deoxyribose mutase or aldolase is missing in this strain. Although no detailed studies were made to analyse the decomposition of AdR, preliminary observations pointed to a probable phosphorolysis by *B. anthracis*.

Cultivation experiments with mutant *thy* suggested the presence of trans-*N*-deoxyribosylase in *Bacillus anthracis* (Ivánovics, 1964). Since no phosphorylitic breakdown of thymidine could be detected in *B. anthracis*, the conversion of thymine to thymidine in the presence of deoxyribonucleosides could only be related to a deoxyribosyl transfer reaction. Indeed, tritiated thymine was converted into thymidine by washed bacterial suspensions in presence of unlabelled pyrimidine deoxyribonucleosides, whereas purine deoxyribonucleosides did not function as substrates in a transfer reaction. The deoxyribosyl transfer reaction appears to be specific as it is known under certain experimental conditions (Roush & Betz, 1958) and assay systems (Zimmerman & Seidenberg, 1964). Although evidence was secured for the existence of deoxyribosyl transfer reaction in *B. anthracis*, further studies are needed to identify the presence of nucleoside: purine pyrimidine deoxyribosyltransferase in the organism.

It is hard to give a satisfactory explanation of increased thymine uptake by *Bacillus anthracis* in the presence of UdR and AdR, although phosphorolytic breakdown of these deoxyribonucleosides was either proved or rendered presumable. Secondary mutation in *B. anthracis* resulting in a block in the catabolism of deoxyribose which in turn increased the availability of intracellular deoxyribose donors rendered the bacteria capable of utilizing low concentrations of exogenous thymine as is known for some Enterobacteriaceae. Nevertheless, there are several points which do not allow a similar conclusion which is valid for Enterobacteriaceae as to the incorporation of thymine. These contradictory facts are as follows. (1) The lack of thymidine phosphorylase in *B. anthracis* does not allow the conversion of the accumulated deoxyribose-1-phosphate plus thymine to thymidine. (2) Exogenous thymidine prevents thymine uptake by either prototrophic or mutant bacteria, although thymidine itself is a deoxyribose donor in transfer reaction in resting bacteria. (3) In spite of that AdR did not serve as a deoxyribosyl donor in transfer reaction in resting bacteria, AdR

highly increased the thymine incorporation in growing bacteria. The facts listed above obscure the mechanism of thymine incorporation in *B. anthracis*, therefore further data are needed for its complete explanation.

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