PYRIMIDINE METABOLISM IN PBS 1-INFECTED

BACILLUS SUBTILIS

PYRIMIDINE METABOLISM IN PBS 1-INFECTED *BACILLUS SUBTILIS*

by

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SCOPE AND CONTENTS:

The pathways in the metabolism of pyrimidine bases, nucleosides and nucleotides in *Bacillus subtilis* were determined and the relative importance of the pathways in the synthesis of dTTP was evaluated.

Pathways involved in the metabolism of pyrimidine compounds in PBS 1-infected cells of *B.subtilis* were proposed on the basis of the present results and those of earlier studies. The contribution of the various pathways to the biosynthesis of dUTP in PBS 1-infected cells was investigated by labelling the phage DNA with radioactive bases and nucleosides in mutant strains blocked at various steps of the metabolism.

PREFACE

The experiments described in this thesis were carried out in the Department of Biology, McMaster University, from September 1970 to August 1974. Except where others are specifically mentioned, this thesis consists entirely of my own original work. No similar thesis has been submitted to any other university.

I am indebted to my Supervisor, Dr. I. Takahashi, for his guidance and support. I would like to thank Mr. M. Botos for always providing clean glassware, Mrs. D. Bradford for assistance in the work and Mr. M. Levine for helpful discussion. I would like to thank the people of Canada, and specifically those of Ontario for support in the form of grants. I thank the National Research Council for post graduate scholarships awarded to me. I would also like to thank Mrs. Patricia Boyd for excellent typing.

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CHAPTER I

INTRODUCTION

Transducing phage PBS 1 for Bacillus subtilis has been isolated in 1961 by Takahashi (128). Since then phages similar to PBS 1 have been isolated by a number of investigators. They include phages 3 NT (56), AR 9 (13, 141) and I 10 (114). PBS 1 is distinct from other transducing phages such as SP10 and SP15 (134, 136, 140). Phage PBP 1 which mediates transduction in Bacillus pumilus also has been found to be distinct from PBS 1 (78). Phage PBS 1 mutates frequently to a clear plaque type called PBS 2 (129). This clear plaque mutant is also able to mediate transduction in B.subtilis, although the frequency of transduction is lower than that of PBS 1 (129).

The morphology of PBS 1 is very complex (32). The phage probably belongs to "type A phages" according to the classification of Bradley (20). PBS 1 has an icosahedral head, a tail core, a collar, a base plate and a striated contractile sheath. Unusual structures like contraction fibres of unknown function, which emerge from the sheath on contraction, and three helical attachment fibres per virion are also present (32). The primary adsorption site of PBS 1 is the flagellum

of host cells (38, 58, 112). According to Raimondo et al. (112) the helical attachement fibres wind around the bacterial flagellum upon adsorption. The interaction between PBS 1 and its host is known as pseudo-lysogenic or carrier state (129). The genome of PBS 1 can be incorporated into spores of B.subtilis, when cells are infected with PBS 1 in late exponential phase or early sporulating stages (130). The ability of PBS 1 to establish pseudo-lysogenic cells or the ability to be incorporated into spores may be explained by induction of a DNase inhibitor in PBS 1-infected cells. This inhibitor acts specifically on a DNase which hydrolyses native PBS 1 DNA (137).

Transducing particles with a range of densities in isopycnic cesium chloride gradients have been reported by Mahler et al.(82). However, Yamagishi and Takahashi (156) have obtained two distinct bands corresponding to transducing particles and plaque-forming particles in isopycnic cesium chloride gradients. Electron microscopic examination has revealed that other bands reported by Mahler et al.(82) contained damaged phage particles and varying amounts of bacterial debris (156).

The molecular weight of the PBS 1 genome is estimated to be 1.9×10^8 (55). It has been reported that host DNA is not degraded extensively after PBS 1 infection and large fragments of host DNA may be incorporated into transducing particles (101, 137). The molecular weight of the host DNA

extracted from transducing particles is heterogenous and ranges from 4×10^6 to 1.3×10^8 (156).

The G+C base composition calculated from the T_m of PBS 1 DNA differs from that calculated from the buoyant density in cesium chloride gradients. This discrepancy is caused by the presence of uracil in the DNA of PBS 1 (132). The chemical analysis shows that PBS 1 DNA contained 28 % G+C. It is demonstrated that PBS 1 DNA has the normal beta-configuration in solution even though it contains uracil in place of thymine (71). Native PBS 1 DNA molecules contain two single strand interruptions per strand (155). The heterogeneity in size of PBS 1 DNA molecules in alkaline sucrose gradients indicates that these nicks are randomly distributed (155). In contrast, the nicks observed in the T5 genome are located at definite positions in only one of the two strands (23).

Price and Cook (104) have shown that after PBS 1 infection a new DNA polymerase is induced. The observation that the development of PBS 1 is resistant to 6-hydroxyphenylazo-uracil and nalidixic acid, which inhibit host DNA synthesis, suggests that the phage induced DNA polymerase may be resistant to these drugs (107, 108). Hydroxyurea inhibits phage DNA synthesis as well as host DNA synthesis (108).

Actinomycin D, an inhibitor of RNA polymerisation, prevents the development of phage PBS 1 (109, 111, 115). However, rifamycin, which also inhibits the synthesis of RNA, does not interfere with the development of PBS 1 (109, 115). Many coliphages such as T4 (49), T5 (12) and P1 (88) are inhibited by rifamycin throughout their development cycle. Phages for B.subtilis such as SP01 (42), β-22 (51), SPP1 (86) and φ-29 (120) and the Bacillus megaterium phage α (127) are also reported to be sensitive to this antibiotic. The development of coliphage T7 becomes resistant to inhibition by rifamycin between 3 and 5 min after infection, due to the synthesis of a phage coded rifamycin-insensitive RNA polymerase (25). The development of PBS 1 is resistant to rifamycin from the beginning of infection. Thus, this phage may introduce a protein during infection that either itself is a rifamycin-insensitive RNA polymerase or that modifies host RNA polymerase to become rifamycin resistant (115). Recently the presence of a rifamycin-insensitive RNA polymerase has been demonstrated in PBS 1-infected cells of B.subtilis (26, 110).

Genetic studies of PBS 2 have been initiated in our laboratory by M. Herrington (52). The substitution of thymine by uracil in the DNA allows the incorporation of 5-fluorodeoxyuridylate into the DNA of PBS 2 (80). Later this analogue has been found to be mutagenic for the phage (52, 53). A number of temperature-sensitive mutants of PBS 2 have been analysed and assigned to ten complementation groups. These

mutants have been located on a linear map by two factor crosses (52).

Fragments of PBS 1 DNA with size of about 1/100 th. of the genome are distributed in a wide band in cesium sulphate-mercury gradients (Yamagishi, unpublished results). This indicates that these fragments are heterogenous in base composition. To test the biological activity of the fragments, a method for phage transformation has been developed (52).

DNA polymerase induced by PBS 1 as well as DNA polymerase I of B.subtilis can utilize both dUTP and dTTP as precursors for DNA (98, 104). A similar situation occurs with DNA polymerase induced by T-even phages of Escherichia coli which can utilize deoxyhydroxymethylcytidine triphosphate (dHMCTP) and dCTP (151, 158) and with DNA polymerase induced after $\varphi\epsilon$ or SP01 infection of B.subtilis, which can use dTTP and deoxyhydroxymethyluridine triphosphate (dHMUTP) (75, 158). The lack of specificity of these phage induced DNA polymerases necessitates the removal of normal host deoxypyrimidine triphosphates from the cell before the onset of phage DNA synthesis. For example, dTTP must be eliminated from PBS 1-infected cells before phage DNA synthesis starts. The enzymes involved in the elimination of one of the host deoxypyrimidine triphosphates and the synthesis of a new phage specific deoxypyrimidine triphosphate are illustrated in the following examples.

The mechanism of the replacement of cytosine by hydroxymethylcytosine (HMC) in the DNA of T-even phages for E.coli (154) has been the subject of a large number of studies.

As early as 5 min after infection with T-even phages a dCTPase activity is found in the infected cells (68, 69, 125). This enzyme converts dCTP to dCMP and thus the host DNA precursor dCTP is removed from the infected cell (68). The precursor for phage DNA synthesis, dHMCTP, is synthesized at the nucleotide level and not after polymerisation of the DNA. Two enzymes involved in the biosynthesis of dHMCTP can be identified in the phage-infected cells. These are dCMP hydroxymethylase (36, 37), which converts dCMP to dHMCMP and dHMCMP kinase (69, 125), which converts dHMCMP to dHMCTP. In addition, a large number of other enzymes and proteins involved in the synthesis of DNA precursors are induced after T-even phage infection. These include dGMP kinase (68), dCMP deaminase (66), dUTPase and dUDPase (45), dTMP synthetase (7, 27), dTMP kinase (68), dihydrofolate reductase (48, 57), ribonucleoside diphosphate reductase (157) and thioredoxin (123). Phage T4 also codes for a TdR kinase (24, 54). Moreover, the catabolism of deoxyribose-1-phosphate is inhibited after T4 infection, so that exogenous thymine can be utilised more efficiently (87).

Degradation of host DNA by phage induced nucleases is another source of deoxyribonucleotides for phage DNA synthesis in cells infected by T-even phages (144, 145). Glucosylation of the dHMCMP residues in the DNA of the T-even phages by glucosyl transferase (68) takes place after DNA is polymerized by a phage induced DNA polymerase (4).

A number of phages contain hydroxymethyluracil (HMU) in place of thymine. These include $\varphi\epsilon$ (118), SP8 (61), SP5 (22, 61), 2C (102) and SP01 (99), which are active on B.subtilis and phage GSW (14) active on B.megaterium. The enzymes involved in the replacement of thymine by HMU have been studied in detail.

In order to eliminate dTTP from infected cells dTTPase (14, 105, 117), dTMPase (3, 95, 117) and an inhibitor for thymidylate synthetase are induced (50). All HMU containing phages active on B.subtilis induce dCMP deaminase (50, 60, 95). In B.megaterium cells infected with phage GSW, dCMP deaminase is absent (14).

In the DNA of phage φ-W-14 which is active on Pseudomonas acidovorans 50 % of the thymine is replaced by 5-(4-aminobutylaminomethyl)-uracil (70). The enzymes involved in this base alteration have not yet been described (70). In the DNA of SP15 for B.subtilis 50 % of thymine is replaced by 5-(4,5 dihydroxypentyl)-uracil (84, 89). Neubert and Marmur (89) suggest that dUTP may be the precursor for phage SP15 DNA synthesis and that uracil in the DNA may be converted to thymine and 5-(4,5 dihydroxypentyl)-uracil after DNA is polymerized. The enzymes involved in the modification of bases in SP15 DNA have not yet been studied.

In PBS 1-infected cells, enzymes which cause the removal of thymidine nucleotides are dTTPase (139) and dTMPase (59, 139). The latter enzyme has been characterized by Price and Fogt (106) and has been shown to have dTMPase as well as

dUMPase activity. It may be involved in the removal of both substrate and product of thymidylate synthetase in PBS 1-infected cells.

In the bacteriophage systems studied so far, base alterations involve the synthesis of a new pyrimidine deoxyribonucleotide by phage coded enzymes and the elimination of one of the deoxyribonucleotide precursors for host DNA synthesis. The base alteration in PBS 1 is unique in that no new deoxypyrimidine compound is required. Deoxyuridine nucleotides are present as intermediates in thymidine biosynthesis in uninfected cells. PBS 1-induced dUMP kinase may thus have a dual role (59). Firstly, it may remove the substrate for dTMP synthetase (59) and secondly, it may play a role in the synthesis of dUDP and dUTP in PBS 1-infected cells (59).

Tomita and Takahashi (138) have shown that a novel enzyme, dCTP deaminase, is induced after PBS 1 infection. This enzyme converts dCTP to dUTP and thus it may play an important role in the synthesis of dUTP in PBS 1-infected cells. The same authors suggest that ribonucleotide reductase, which catalyses the reduction of ribonucleotides to deoxyribonucleotides, may play a role in the biosynthesis of dUTP (138). Other enzymes such as CdR deaminase and UdR kinase may also be involved in the synthesis of dUTP (138). The same authors (138) suggest that dCTP deaminase may be the major pathway for the formation of dUTP in PBS 1-infected cells,

since the specific activity of this enzyme is much higher than those of UDP reductase, CdR deaminase and UdR kinase.

Evaluation of the importance of various pathways in the biosynthesis of dUTP in PBS 1-infected cells requires thorough knowledge of the pathways of the host pyrimidine metabolism and changes which occur after phage infection. However, very little is known about the metabolism of pyrimidine compounds in B.subtilis at present.

In E.coli, Salmonella typhimurium and Saccharomyces cerevisiae uracil is converted to UMP by an enzyme called UMP pyrophosphorylase (9, 21, 47). Mutants lacking this enzyme (upp) have been isolated in E.coli, S.typhimurium and S.cerevisiae (9, 21, 47). These upp mutants are resistant to fluorouracil (9, 21) (See Fig.1).

In enteric bacteria and S.cerevisiae two reactions are known to be involved in the metabolism of UR. This nucleoside can be converted to UMP by UR kinase or alternatively, it can be degraded to uracil and ribose-1-phosphate by UR phosphorylase (9, 97). Mutants lacking UR kinase (udk) or UR phosphorylase (udp) have been isolated in S.typhimurium and S.cerevisiae (9, 47). Mutants blocked in either of these two enzymes are sensitive to fluorouridine, but double mutants of type udk udp are resistant to this analogue (9, 47, 97). These two enzymes are absent in Acinetobacter calcoaceticus, and consequently this bacterium can not metabolise UR (100). B.subtilis can metabolize UR since radioactive UR can be incorporated into DNA and RNA (6, 19). More than 90 % of the

radioactivity incorporated into nucleic acids is in RNA and only a small fraction of the radioactivity if found in the DNA fraction (6).

UdR can be converted to dUMP by TdR kinase or it can be degraded to uracil and deoxyribose-1-phosphate by TdR phosphorylase in enteric bacteria (9, 34, 97). Since UR phosphorylase seems to be able to degrade FUdR to fluorouracil at low rates, only mutants lacking TdR kinase, TdR phosphorylase as well as UR phosphorylase are resistant to FUdR (9). Figure 1 summarizes the metabolism of uracil, UR and UdR in enteric bacteria (9).

At present only one reaction is known in the metabolism of cytosine. In enteric bacteria and in S.cerevisiae this base is deaminated by a specific cytosine deaminase (9, 21, 47, 97). Pyrimidine requiring mutants of E.coli and S.typhimurium can grow on cytosine as their sole source of pyrimidine. Mutants lacking cytosine deaminase (cod) can no longer grow with cytosine as sole source of pyrimidine and these mutants are resistant to fluorocytosine (9, 47). Cytosine does not support the growth of strains which require CR for growth (pyrG mutants). This indicates that there is no CR phosphorylase or CMP pyrophosphorylase active in enteric bacteria (91).

In enteric bacteria CdR and CR are deaminated by an enzyme, deoxycytidine-cytidine deaminase (9, 63, 97). Mutants lacking this enzyme (cdd) in E.coli and S.typhimurium are sensitive to FUdR and resistant to FCdR (9, 97). These strains

can no longer use CdR or CR as sole source of pyrimidine (9).

The observation that cdd mutants in enteric bacteria are resistant to FCdR indicates that there are no other reactions which metabolize FCdR or CdR. The inability of enteric bacteria to incorporate radioactive CdR into nucleic acids indicates the absence of CdR kinase (64, 190). In B.megaterium a CdR kinase is present (142) and its presence in B.subtilis has been suggested by the observation that B.subtilis can use CdR as a precursor for DNA synthesis (8, 115).

The existence of CR requiring strains of S.typhimurium and of E.coli indicates that CR can be converted to CMP, CDP and CTP. This reaction is mediated by UR kinase in enteric bacteria (9, 91, 97). No kinase specific for CR has been found as yet.

The isolation of thymine requiring mutants in a large variety of bacteria indicates that salvage pathways must exist which are capable of converting added thymine or TdR to dTMP and dTTP (97). In enteric bacteria, two enzymes are involved in the metabolism of thymine and TdR. Firstly, TdR can be converted to dTMP by TdR kinase. Mutants blocked in this enzyme (tdk) are incapable of phosphorylating both TdR and UdR (97). A second enzyme is TdR phosphorylase which acts both in the conversion of thymine to TdR and in the reverse reaction. This enzyme metabolises TdR as well as UdR (97). Mutants lacking this enzyme (tpp) are unable to use either TdR or UdR

as sole carbon source (97). These two enzymes are present in B.subtilis, although thymine is claimed to be not normally involved in the synthesis of thymidine nucleotides in B.subtilis (6, 19, 116). In P.acidovorans and in Lactobacilli TdR phosphorylase seems to be absent (67, 143). Both TdR phosphorylase and TdR kinase are absent in A.calcoaceticus (100). As a consequence, it is not possible to isolate thymine requiring mutants blocked in dTMP synthetase in A.calcoaceticus. Pyrimidine requiring mutants of bacterial species tested so far cannot grow on thymine or thymidine as sole source of pyrimidine (97). This indicates that there are no enzymes capable of converting thymine or TdR to other pyrimidine compounds in bacteria. On the other hand, Neurospora crassa seems to be able to convert thymine and TdR to uracil (35).

Trans-N-deoxyribosylase, which catalyses the transfer of deoxyribose from one base to another without intermediate synthesis of deoxyribose-1-phosphate, is found in Lactobacilli (11). This enzyme is absent in enteric bacteria (97).

The de novo biosynthetic pathway of pyrimidine compounds has been studied in many organisms (97). The end product of this pathway is UMP in all cases. Mutants blocked in each step of the conversion of aspartate and carbamyl-phosphate to UMP (pyrA, B, C, D, E and F) have been isolated in E.coli and S.typhimurium (97). In B.subtilis pyrA, B, C, D, E and F mutants are known and the mutations are found to be clustered (30). In E.coli and S.typhimurium on the other hand,

these mutations appear to be unlinked (119, 133). A mutant which is affected in the conversion of UMP to UDP (pyrH) is isolated in S.typhimurium (9). Mutants which are incapable of converting UTP to CTP (pyrG) and require CR for growth have been isolated in S.typhimurium (9). The amination of uridine ribonucleotides takes place at the triphosphate level by CTP synthetase in all organisms studied so far (97). The reverse reaction, deamination of cytidine ribonucleotides is not known (97) although cytidine deoxyribonucleotides are deaminated in enteric bacteria (93). A dCTP deaminase activity was found in crude extracts of S.typhimurium (93). The presence of this enzyme provides an explanation for the original observation of Neuhard (90) that 80 % of the thymidine nucleotides in this organism are derived from cytidine ribonucleotides via cytidine deoxyribonucleotides and uridine deoxyribonucleotides. An E.coli strain lacking dCTP deaminase (paxA) has been described (96). In Lactobacilli, a dCMP deaminase instead of a dCTP deaminase is found (121). In enteric bacteria the product of dCTP deamination, dUTP, is degraded rapidly by dUTP pyrophosphorylase to dUMP (46), which is the substrate for dTMP synthetase. This dUTP pyrophosphorylase has generally been considered responsible for the absence of dUMP in the DNA of E.coli (46).

Thymidine nucleotides are made in E.coli by thymidylate synthetase (39). Methylene tetrahydrofolate is the methyl group donor in this reaction (39). By selecting clones resistant to the folate antagonists, aminopterin and

trimethoprim in the presence of thymine, thymine requiring mutants have been obtained in many bacterial species (97). These mutants are blocked in dTMP synthetase (thyA). When the dTMP synthetase reaction is blocked, tetrahydrofolate remains available for the other reactions requiring this compound. For this reason thyA mutants are resistant to the effect of folate antagonists (150).

In B.subtilis Wilson et al. (150) and Anagnostopoulos and Schneider (2) have shown that two unlinked genetic loci, thyA and thyB (or thyY and thyX) govern the synthesis of thymidine nucleotides. A mutation at both sites, thyA thyB, is necessary to produce the thymine dependent phenotype. The thyA locus in B.subtilis codes for dTMP synthetase (150). The thyB locus codes for an unknown enzyme. Strains which are thyA$^+$ are sensitive to inhibition by aminopterin (150). It is not known whether the thyB locus exists in enteric bacteria. If so, the thyA and thyB loci must be closely linked, since the requirement for thymine can be transduced into wild type strains of S.typhimurium by phage P22 and into strains of E.coli by phage P1 (1, 31). The hypothesis that the thymine requirement in S.typhimurium is a result of two mutations at two closely linked loci, is supported by genetic data obtained by Eisenstark et al. (31). Transduction of thy$^-$ character with an outside marker into a recipient strain would require co-transfer of three loci, while the transduction of thy$^+$ character with an outside marker would require co-transfer of only one of the thy loci and the outside marker.

The latter would be therefore expected to occur more frequently. Data obtained by Eisenstark *et al*. (31) show that co-transduction of *thy*$^+$ character with an outside marker indeed occurs more frequently than that of *thy*$^-$ character. In *S.typhimurium* thymine independent revertants which are still resistant to aminopterin have been obtained (31). They may be similar to the *thyA thyB*$^+$ strains of *B.subtilis* (150).

Alikhanian *et al*. (1) observed that in *E.coli* 64 out of 134 *thy* mutants were located at one site, while the others were distributed over 16 other sites. These 64 mutants showed a temperature-sensitive thymine dependence and they mapped to the left of all other *thy* mutants investigated (1).

Förster and Holldorf (quoted in 97) suggested that the second pathway for dTTP synthesis may involve direct methylation of dCTP, followed by deamination of 5-methyl-dCTP to dTTP. Although this reaction favours the hypothesis that there are two independent pathways involved in the synthesis of thymidine nucleotides, the suggested reaction is not consistent with the results obtained with aminopterin. Both dTMP synthetase and the reaction proposed by Förster and Holldorf require methylene tetrahydrofolate as the methyl group donor and therefore *thyA thyB*$^+$ strains as well as *thyA*$^+$ *thyB* strains are expected to be sensitive to folate antagonists. In fact, *thyA thyB*$^+$ strains are found to be resistant to aminopterin (150).

In all organisms studied so far, deoxyribonucleotides are synthesized by reduction of ribonucleotides by ribonucleotide reductase (5, 97). The reduction of ribonucleotides is the exclusive pathway for the biosynthesis of deoxyribonucleotides (8, 40, 41, 65). Ribonucleotide reductase has been studied in detail in E.coli and in Lactobacillus leichmannii. All four ribonucleoside diphosphates are reduced by the same enzyme in E.coli (72, 113). This reduction is coupled with oxidation of the reduced form of thioredoxin, a low molecular weight protein, that acts as the hydrogen donor in this reaction (74).

The enzyme in E.coli is an agglomerate of two proteins, B1 and B2 (135). The molecular weight of the ribonucleotide reductase in E.coli is 245,000 (135). The ribonucleoside diphosphate reductase induced after infection of E.coli with phage T4 is similar to the E.coli enzyme in overall structure (15). However, T4 thioredoxin seems to be substantially different from that of E.coli (124). In L.leichmannii a ribonucleoside triphosphate reductase is found which is dependent on coenzyme B12 (43). The molecular weight of this enzyme is estimated to be 110,000 (43).

The enzymes from E.coli and L.leichmannii differ not only in substrates but also in sensitivity to hydroxyurea (33). The L.leichmannii enzyme is insensitive to hydroxyurea, while the E.coli enzyme is inhibited both in vivo and in vitro by this drug (122). The inhibition of the E.coli ribonucleotide reductase is associated with the B2 subunit, which binds a

non-heme iron cofactor (122).

The growth of B.subtilis is inhibited by hydroxyurea (8). This inhibition can be overcome by addition of deoxyribonucleosides to the medium (8). A mutant which is affected in ribonucleotide reduction (tsA13) shows increased sensitivity to hydroxyurea (8). A similar observation has been made with a mutant of E.coli affected in ribonucleoside diphosphate reductase (nrd) (40). A temperature-sensitive DNA mutant of E.coli (dnaF) has been shown to have a temperature-sensitive B1 subunit of ribonucleotide reductase (41).

This thesis is devoted to a quantitative evaluation of the contributions of various pathways to the synthesis of dUTP in PBS 1-infected cells of B.subtilis. Such an evaluation requires thorough knowledge of the pathways involved in the synthesis of this compound. Since the pyrimidine metabolism of the host cells was virtually unknown, the first part of this thesis is devoted to an investigation of the pathways of pyrimidine metabolism in uninfected B.subtilis.

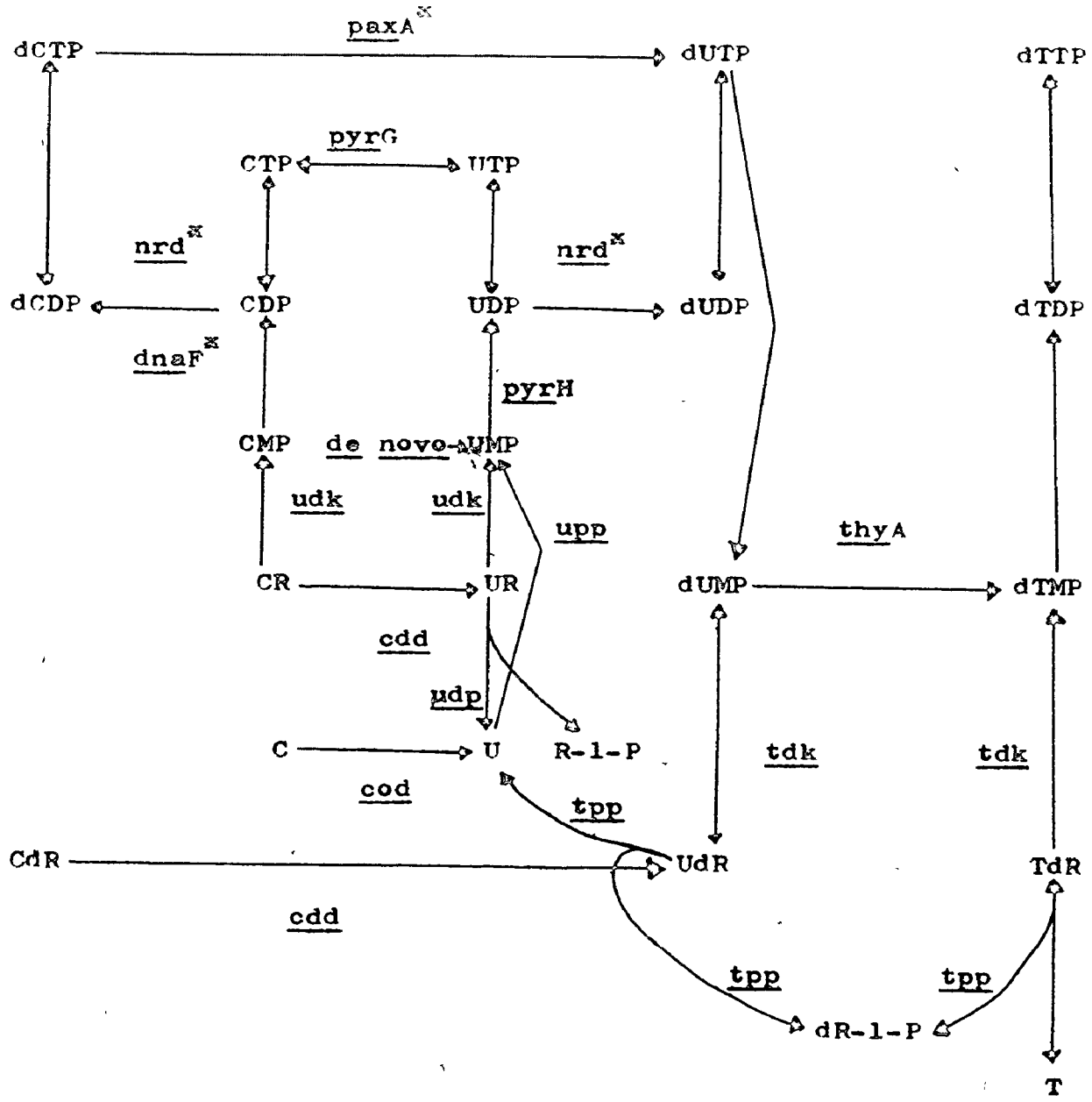
The second part is devoted to the synthesis of dUTP in PBS 1-infected cells. According to Tomita and Takahashi (137, 138), deamination of dCTP may be the major pathway for the formation of dUTP in PBS 1-infected cells, since the specific activity of dCTP deaminase is much higher than that of other enzymes possibly involved in the synthesis of deoxyuridine nucleotides. The same authors suggest that the real evaluation of the role of dCTP deaminase should be made

by a comparison of the following two pathways: UDP $\longrightarrow$ dUDP $\longrightarrow$ dUTP and CDP $\longrightarrow$ dCDP $\longrightarrow$ dCTP $\longrightarrow$ dUTP.

A comparison of the specific activities of the enzymes involved in these pathways would not permit evaluation of their relative importance in the synthesis of dUTP in PBS 1-infected cells, since the specific activities of enzymes *in vitro* are sometimes not related to the actual metabolic flux *in vivo* (152). Therefore, in order to evaluate the contributions of the various pathways involved in the synthesis of dUTP, *in vivo* labelling experiments have been carried out with infected cells of mutants of *B.subtilis* blocked in various steps of pyrimidine metabolism. A comparison of the specific radioactivities of dUMP and CMP in the phage DNA allows an evaluation of the relative importance of the pathways involved in the synthesis of dUTP in PBS 1-infected cells of *B.subtilis*.

Figure 1

Pathways of biosynthesis of pyrimidine nucleotides in *Salmonella typhimurium* (9).



* Mutations only known in *E.coli*.

CHAPTER II

MATERIALS AND METHODS.

1. Symbols and abbreviations.

Symbols to designate the genotype of mutant bacteria were according to the proposals of Demerec *et al*. (29).

The units of length, weight, volume and time are abbreviated as in the Journal of Bacteriology. The abbreviations used for bases, nucleosides, nucleotides and analogues were those used in the Journal of Bacteriology:

T	thymine
C	cytosine
U	uracil
HMU	hydroxymethyluracil
HMC	hydroxymethylcytosine
dHMUMP	deoxyhydroxymethyluridine monophosphate
dHMUTP	deoxyhydroxymethyluridine triphosphate
dHMCMP	deoxyhydroxymethylcytidine monophosphate
dHMCTP	deoxyhydroxymethylcytidine triphosphate
CdR	deoxycytidine
CR	cytidine
UdR	deoxyuridine
UR	uridine
TdR	thymidine
AdR	deoxyadenosine
GdR	deoxyguanosine
NdR	AdR+GdR+CdR+TdR.
FUdR	5-fluorodeoxyuridine
FUR	5-fluorouridine
FCdR	5-fluorodeoxycytidine
FCR	5-fluorocytidine

Other abbreviations used were:

PFU	plaque-forming unit
MOI	mutiplicity of infection

AD	adsorption medium
PA	penassay broth
TBB	Tryptose Blood Agar Base.
R-1-P	ribose-1-phosphate
dR-1-P	deoxyribose-1-phosphate
TCA	trichloroacetic acid
CPM	count per minute
EDTA	ethylenediaminetetraacetic acid.

Symbols to designate genotypes used were:

pyrA, B, C, D, E, F	Mutants blocked in a step of the de novo synthesis of UMP
pyrG	mutant requiring CR, lacking CTP synthetase
pyrH	mutant affected in UMP kinase
upp	mutant lacking UMP pyrophosphorylase
udk	mutant lacking UR kinase
udp	mutant lacking UR phosphorylase
tpp	mutant lacking TdR phosphorylase
tdk	mutant lacking TdR kinase
cod	mutant lacking cytosine deaminase
cdd	mutant lacking deoxycytidine-cytidine deaminase
thyA	mutant lacking dTMP synthetase
thyB	mutant lacking unknown enzyme in dTMP synthesis
tsA	temperature-sensitive DNA mutant in B.subtilis
dnaF	temperature-sensitive DNA mutant in E.coli
nrd	mutant lacking ribonucleotide reductase
dns	mutant requiring deoxyribonucleosides
dck	mutant lacking CdR kinase
crk	mutant lacking CR kinase
ddd	mutant lacking dCDP deaminase
fur	mutant resistant to 5-fluorouracil
paxA	mutant lacking dCTP deaminase

2. Bacteriophage and bacterial strains.

Bacteriophage PBS 1 was from our laboratory stock. Phage lysates were prepared from a single plaque isolate of PBS 1. Phage PBS 1 was maintained in spores of B.subtilis as described by Takahashi (130). DNA negative mutants of PBS 2, SFU50 and SNG5, were provided by M.Levine.

The bacterial strains used in this study are listed in Table 1.

Table 1.

List of Bacterial strains

Strain	Source
SB19E (str^r ery^r)	Laboratory stock
A26 pyr-1	Laboratory stock
167 tsA$^+$ thyA thyB	D. Karamata (8)
167 tsA13 thyA thyB	D. Karamata (8)
3046 (fur)	M. Polsinelli
SB19E pyr-2	New isolate
SB19E pyr-3	" "
SB19E pyr-2 cdd-1	" "
SB19E pyr-2 cdd-1 dck-1	" "
SB19E pyr-2 cdd-1 dck-2	" "
SB19E pyr-2 cdd-1 dck-5	" "
SB19E pyr-2 cdd-1 ddd-1	" "
SB19E pyr-2 cdd-1 ddd-2	" "
A26 pyr-1 dns-1	" "
SB19E dns-2	" "
SB19E dns-3	" "
SB19E dns-4	" "
SB19E dns-5	" "
SB19E dns-6	" "
SB19E dns-7	" "
SB19E dns-8	" "
167 tsA13 cdd-1 thyA thyB	" "
167 tsA13 cdd-1 thyA$^+$ thyB	" "
167 tsA13 cdd-1 thyA thyB$^+$	" "
SB19E tsA13 cdd-1	" "
SB19E tsA13 cdd-1 dck-3	" "
SB19E tsA13 cdd-1 ddd-3	" "
SB19E tsA13 cdd-1 pyrG1	" "
SB19E tsA13 cdd-1 pyrG1 dck-4	" "
SB19E tsA13 cdd-1 dck-3 crk-1	" "
SB19E tsA13 cdd-1 dck-3 crk-2	" "
SB19E tsA13 cdd-1 ddd-3 crk-3	" "
SB19E tsA13 cdd-1 ddd-3 crk-4	" "

3. Culture Media.

a) Difco Penassay Broth (PA).

b) Minimal medium (MMC) was the minimal medium described by Spizizen (126), to which Casamino Acids (Difco) was added at 0.02 %.

MMC contained:

$(NH_4)_2SO_4$	2.0 g
K_2HPO_4	14.0 g
KH_2PO_4	6.0 g
sodium citrate	1.0 g
$MgSO_4.7$ aq.	0.2 g
distilled H_2O	1.00 liter

The pH was adjusted to pH 7.0. After sterilisation 25 ml of a 20 % glucose solution were added and 2 ml of a 10 % solution of Casamino Acids (Difco). MMC agar contained 16 g agar (Difco) per liter of medium. Bases and nucleosides were added at a final concentration of 10 µg/ml when required.

c) Adsorption medium (AD) was used for phage dilutions and phage adsorption (14).

d) Difco Tryptose Blood Agar Base (TBB).

e) Transformation medium BGM.

A modification of the BGM medium described by Mahler (81) was used.

To 100 ml of autoclaved Spizizen's minimal medium (126) the following solutions were added:

5 ml	10 % glucose
1 ml	10 % Difco Yeast Extract
0.2 ml	10 % Difco Casamino Acids
1 ml	0.5 % thymine

Prior to use 1 ml of 10 % arginine was added.

f) Competence medium CM.

A modification of the CM medium described by Mahler (81) was used.

To 100 ml of autoclaved Spizizen's minimal medium (126) the following solutions were added;

5 ml	10 % glucose
1 ml	10 % Difco Yeast Extract
0.2 ml	10 % Difco Casamino Acids
0.1 ml	0.5 % thymine

Prior to use the following supplements were added:

2.5 ml	0.125 M $CaCl_2$
2.5 ml	0.1 M $MgCl_2$
1.0 ml	0.05 M spermine HCl (pH 8.8)

4. <u>Phage techniques</u>.

The assay for phage PBS 1 was carried out as described previously (129) except that AD medium was used for phage dilution and adsorption (156).

Phage lysates prepared according to Takahashi's method (129) were centrifuged at 3000 x g for 15 min to remove cell debris. The supernatant fluid was centrifuged at 40,000 x g for 60 min. The pellet was resuspended in buffer containing 0.02 M Tris-Cl (pH 7.5), 0.1 M sodium chloride and 0.01 M magnesium sulphate and incubated with lysozyme (100 µg/ml), DNase (10 µg/ml) and RNase (10 µg/ml) at 37 C for 30 min. The phage particles were pelleted again by centrifugation at 40,000 x g for 60 min and they were resuspended in 1x SSC (0.15 M sodium chloride + 0.015 M sodium citrate).

Phage particles were further purified by isopycnic cesium chloride gradient centrifugation as described by Yamagishi and Takahashi (156). The banded phage particles were removed from the centrifuge tube with a syringe. The phage was diluted five-fold with PA and stored at 4 C. The phage was stable for many months under this condition. The titers of these preparations were usually 2 to 5 x 10^{11} PFU/ml.

5. <u>Bacterial transformation</u>.

Bacterial transformation was carried out as described by Mahler (81). The recipient cells were grown for 4 h in BGM and then transferred to CM. Bacterial DNA extracted according to the procedure of Takahashi (131) was added at a final concentration of 1 µg/ml.

6. Mutagenesis.

A culture of B.subtilis grown in PA for 4 h was mixed with an equal volume of a concentrated solution of N-methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine) in water to give a final concentration of about 50 µg/ml. After 10 min at 37 C the mutagenized culture was plated on appropriate selection media. The replica-plating technique described by Lederberg and Lederberg (76) was used in some instances to select desired mutants.

7. Incorporation of radioactive bases and nucleosides into DNA and total nucleic acids.

Broth cultures were obtained by inoculating PA with cells grown overnight on TBB agar. Growth of the cultures was followed with a Klett-Summerson colorimeter equipped with filter # 54 (500-700 nm wavelength). Radioactive nucleosides or bases were added to a culture in late exponential phase (Klett units= 80). At various times 0.2 ml-samples were pipetted into 5 ml of cold 10 % TCA to measure the incorporation of radioactivity into the total nucleic acids. Samples were kept at 0 C for 30 min. The TCA precipitates were collected on membrane filters (pore size: 0.45 µm) by suction and washed three times with cold 5 % TCA. The filters were then dried under an infra-red lamp and the radioactivity was determined by liquid scintillation counting.

In order to measure the incorporation of radioactivity into DNA, 0.2 ml-samples of the culture were pipetted into 0.8 ml of 0.3 N potassium hydroxyde and kept at 0 C for 30 min. The samples were incubated at 37 C for 18 h to hydrolyse RNA. The samples were neutralized with 0.13 ml of 6 N hydrochloric acid and DNA was precipitated with 5 ml of cold 10 % TCA. The samples were kept at 0 C for 30 min and the TCA precipitable radioactivity was measured as described above.

For the incorporation of radioactive bases or nucleosides into PBS 1-infected cells, a similar procedure was used. When a broth culture of B.subtilis reached a turbidity of 80 Klett units PBS 1 was added at an MOI of 4. At various times after infection radioactive compounds were added to cultures.

8. Crude extracts.

Cells of B.subtilis were grown to the late exponential phase in PA and cooled rapidly in ice water. Cells were harvested by centrifugation at 7000 x g for 10 min. The pellets were frozen and stored at -5 C. To obtain crude extracts, cells resuspended in appropriate buffer were treated twice with a French Pressure Cell (American Instrument Co.) at a pressure of 15,000 lbs/ $inch^2$. Unbroken cells and debris were removed by centrifugation at 30,000 x g for 30 min and the supernatant fluid was used as crude extract.

Extracts for enzyme assays in PBS 1-infected cells were made from cells infected as described earlier and harvested between 25 and 30 min after infection.

The concentration of protein in crude extracts was determined by the procedure of Lowry et al. (79) or estimated from the absorbance at 280 nm. An absorbance of 0.8 at 280 nm with a 10 mm light path corresponded to 1 mg/ml protein.

9. Enzyme assays.

All enzyme assays were carried out in crude extracts prepared as described above. Unless otherwise stated, the reactions were terminated by heating the assay mixture at 100 C for 3 min. The resulting precipitates were removed by centrifugation and appropriate amounts of the supernatant fluids, usually 30 µl, were spotted on Whatman # 1 filter paper together with non-radioactive reference nucleotides, nucleosides and bases. Radioactive products were separated from substrates by paper chromatography. The amount of product formed in the reaction mixture was obtained from the % of total CPM on the chromatogram, present in the product spots. At least once for the assay of each of the enzymes, the stability of both substrates and products was investigated by determining the distribution of radioactivity in bases, nucleosides and nucleotides. The total CPM recovered from the chromatograms did not change after incubation.

Therefore, all radioactivity was accounted for and the amount of product formed in the reaction mixture could be determined by comparison of the CPM in the product spot(s) and the total CPM on the chromatogram. Enzyme activities were corrected for the blank values obtained with boiled extracts.

The reproducibility of assays was generally within 10 % between extracts of one strain, except for assays of dCDP deaminase, UTP- and CTP reductase. The assays of these enzymes will be discussed later.

a) Deoxyuridine kinase or deoxyuridine phosphorylase.

The procedure used was a modification of the one described by Tomita and Takahashi (138). Crude extracts were prepared in 50 mM potassium phosphate buffer (pH 7.4). The reaction mixture contained for both enzyme assays: 50 mM potassium phosphate buffer (pH 7.4) , 1.25 mM ATP, 1.25 mM $MgCl_2$, 0.34 µM 6-^{3}H-UdR (3 µCi) and aliquots of extract corresponding to 0.4 - 0.7 mg protein in a total volume of 1.0 ml. The rate of product formation was constant over a period of 10 min and the initial velocity of the reaction was proportional to the amount of enzyme added upto a rate of 0.4 nmole/h. The assay mixture was incubated for 10 min at 37 C. The products, dUMP for UdR kinase or uracil for UdR phosphorylase, were separated from the substrate by

descending paper chromatography with a mixture of 95 % ethanol: saturated sodium tetraborate: 5 M ammonium acetate: 0.5 M EDTA (220:80:20:0.5 by volume) as solvent.

b) Deoxycytidine-cytidine deaminase.

The procedure was described by Tomita and Takahashi (138). Crude extracts were prepared in 50 mM Tris-Cl buffer (pH 7.5). The reaction mixture contained : 25 mM Tris-Cl buffer pH 7.5, 125 µM $MgCl_2$, 1.0 µM 5-^{3}H-CdR (5 µCi) or 0.25 µM 5-^{3}H-CR (5 µCi) and aliquots of extract corresponding to 0.5 mg protein in a total volume of 1.0 ml. The rate of product formation was constant over a period of 15 min and the initial velocity of the reaction was proportional to the amount of enzyme added upto a rate of 0.8 nmole/h for CdR deamination and 0.21 nmole/h for CR deamination. The assay mixture was incubated for 15 min at 37 C. The product UdR or UR, was separated from the substrate by ascending paper chromatography with a mixture of isopropanol: concentrated hydrochloric acid: water (170:42:28 by volume) as solvent.

c) Deoxycytidine kinase.

The method was similar to the procedure for the UdR kinase assay described by Tomita and Takahashi (138). The assay conditions for this enzyme were not

optimized. Crude extracts were prepared in 50 mM Tris-Cl buffer (pH 7.5). The reaction mixture contained: 50 mM Tris-Cl buffer (pH 7.5), 1.25 mM ATP, 1.25 mM $MgCl_2$, 0.5 µM 5-^{3}H-CdR (2.5 µCi) and aliquots of crude extract corresponding to 0.2 mg protein in a total volume of 1.0 ml. Under these conditions the rate of product formation was constant at least for 15 min. The initial velocity of the reaction was proportional to the amount of enzyme added upto a rate of 0.45 nmole/h. The reaction mixture was incubated at 37 C for 15 min. Products (dCMP+dCDP+dCTP) were separated from the substrate by descending paper chromatography as described in the assay of UdR kinase. More than 80 % of the product was in the dCDP spot.

d) Cytidine kinase.

Crude extracts were prepared in 50 mM Tris-Cl buffer (pH 7.5). The assay mixture was as described for CdR kinase except that 0.25 µM 5-^{3}H-CR (5 µCi) was used as substrate and aliquots of extract corresponding to 0.8 mg protein. The rate of product formation was not linear. The assay was terminated after 3 min at 37 C since the reaction did not proceed any further. Therefore this method could not be used in quantitative determinations of cytidine kinase activity. Products (CMP+CDP+CTP) were separated from

the substrate as described in the CdR kinase assay. The activity was expressed as the amount of product formed after 3 min incubation.

e) Thymidylate synthetase.

The assay of dTMP synthetase was performed by the procedure of Wahba and Friedkin (147).

f) The thyB coded thymidylate synthetase.

The assay procedure was based on the technique described by Lomax and Greenberg (77) for the assay of thymidylate synthetase in E.coli. The release of tritium from the 5-position of dUMP or dUDP was measured. Crude extracts were prepared in 50 mM Tris-Cl buffer (pH 7.5). The reaction mixture contained: 25 mM Tris-Cl buffer (pH 7.5), 250 µM $MgCl_2$, 0.1 µM 5-^{3}H-dUMP or 5-^{3}H-dUDP (1.5 µCi) and aliquots of crude extract corresponding to 2.0-2.7 mg protein in a total volume of 1.0 ml. The mixture was incubated at 37 C. The rate of product formation was not linear. The assay was terminated after 5 min since the reaction did not proceed any further. Therefore this method could not be used in quantitative determinations of the enzyme. The reaction was terminated by adding 0.5 ml of a charcoal slurry (1 g / 50 ml of 10 mM potassium phosphate buffer pH 7.5) to 0.33 ml of reaction mixture. The

charcoal, which adsorbed the unreacted substrate, was removed by centrifugation and 0.5 ml of the supernatant containing tritium released from the 5-position of dUMP or dUDP was mixed with 5 ml of a scintillation fluid (Aquascint II). The results were expressed as CPM released in 0.5 ml of supernatant. A 0.33 ml-sample was mixed with the charcoal slurry without incubation and served as control.

g) Deoxycytidine triphosphate deaminase.

This assay was performed by the procedure described by Tomita and Takahashi (138). Crude extracts were prepared in 50 mM Tris-Cl buffer (pH 7.5).

h) Deoxycytidine diphosphate deaminase.

Crude extracts were prepared in 50 mM Tris-Cl buffer (pH 7.0). The reaction mixture contained: 25 mM Tris-Cl buffer (pH 7.0), 5 mM $MnCl_2$, 334 µM 5-^{3}H-dCDP (2.5 µCi) and aliquots of crude extract corresponding to 0.25-0.70 mg protein in a total volume of 1.0 ml. The rate of product formation was constant for 15 min and the initial velocity of the reaction was proportional to the amount of enzyme added upto a rate of 130 nmoles/h. The reaction mixture was incubated at 37 C for 10 min. The product was separated from the substrate by ascending

paper chromatography as described in the assay of CdR-CR deaminase.

i) Uridine triphosphate reductase.

Crude extracts were prepared in 50 mM potassium phosphate buffer (pH 7.4) containing 0.2 mM mercaptoethanol. The assay mixture contained: 25 mM potassium phosphate buffer (pH 7.4), 1 mM dithiothreitol, 1.2 mM $CaCl_2$, 0.285 µM 5-^{3}H-UDP (5 µCi) and aliquots of crude extract corresponding to 3.5 mg protein in a total volume of 1.0 ml. The rate of product formation was not constant. The velocity of the reaction was proportional to the amount of enzyme added upto a rate of 0.1 nmole/h. Samples were incubated at 37 C. The specific enzyme activity was calculated from the amount of products formed after 10 min. The products (dUDP and dUTP) were separated from the substrate as described in the assay of UdR kinase and UdR phosphorylase.

j) Cytidine triphosphate reductase.

Crude extracts were prepared as in the assay of UTP reductase. The reaction mixture contained: 25 mM potassium phosphate buffer (pH 7.4), 15 mM $MgCl_2$, 30 µmoles of creatine phosphate, 0.05 mg creatine phosphokinase, 0.255 µM 5-^{3}H-CTP (5 µCi) and aliquots of crude extract corresponding to

0.5-1.0 mg protein in a total volume of 1.0 ml. The rate of product formation was not constant. The velocity of the reaction was proportional to the amount of enzyme added upto a rate of 0.1 nmole/h. Samples were incubated at 37 C. The specific activity was calculated from the amount of products formed after 10 min. The products (dCTP and dCDP) were separated from the substrate as described in the UdR kinase and UdR phosphorylase assays.

10. Stoichiometry of dCDP deaminase reaction.

The standard assay for dCDP deaminase was scaled up six-fold and radioactive substrate was omitted. The assay mixture was incubated for 10 or 20 min at 37 C. In addition, the nucleotide composition of a control mixture with boiled extract was determined. The reaction was terminated by heating the assay mixtures at 100 C for 3 min. The resulting precipitates were removed by centrifugation and the supernatant liquid was quantitatively applied onto a Dowex-1-formate column (9 x 1.1 cm). The substrate and products were separated by gradient elution with a concave gradient formed by mixing 1 M ammonium formate (pH 8.2) in a mixing chamber with 250 ml water (83). Twentyfive-drop fractions (about 1.3 ml) were collected and the absorbancy of each fraction was measured at 260 nm. The amount of nucleotide in a peak was estimated from the total absorbancy. Compounds in

the various peaks were identified from acid, neutral and alkaline UV absorption spectra. The molar extinction coefficient at 260 nm for dUMP and dUDP was 10×10^3. The molar extinction coefficient for dCMP and dCDP at 260 nm was 7.4×10^3. The separation of dUDP and dCDP was not complete and the composition of the two nucleotides in this peak was determined by analysing the acid UV absorption spectrum.

11. Labelling of bacterial DNA with radioactive bases and nucleosides.

Cells of B.subtilis were grown in PA to a turbidity of 60 Klett units and labelled with ^{14}C-CR, ^{14}C-CdR, ^{14}C-uracil or 5-^{3}H-UR.

Two min prior to the addition of radioactive material, unlabelled CR and uracil were added to the culture at a final concentration of 2 µg/ml each, to lower the specific activity of the radioactive compounds. Under these conditions less than 10 % of the added radioactivity was incorporated throughout the course of the experiment and, thus, the DNA of the cells was labelled uniformly. When the non-radioactive compounds were absent, all radioactivity was incorporated into nucleic acids in less than 10 min.

Cells were harvested 60 min (2-3 generations) after the addition of radioactive material, by centrifugation at 3000 x g for 10 min. DNA was extracted by the

procedure of Takahashi (131) with minor modifications. Cells were resuspended in 1 x SSC and lysed with lysozyme (100 µg/ml) at 37 C. Sodium laurylsulphate was added at a final concentration of 0.45 % and the lysate was heated at 60 C until lysis was complete. Two volumes of cold 95 % ethanol were added and crude fibres were collected with a glass rod. The fibres were dissolved in 1 x SSC + 0.45 % sodium laurylsulphate. Denatured protein was salted out by adding solid sodium chloride at a concentration of 1 M. The precipitate was removed by centrifugation at 27,000 x g for 30 min at 4 C. Two volumes of cold 95 % ethanol were added to the supernatant fluid. The precipitate was collected by centrifugation at 10,000 x g for 10 min and redissolved in 1 x SSC. Pronase was added at a final concentration of 10 µg/ml and the mixture was incubated at 37 C for 45 min. Potassium hydroxide was then added at a concentration of 0.3 N. After incubation at 37 C for 18 h 200 µg of unlabelled carrier DNA was added. The solution was neutralized with concentrated hydrochloric acid and DNA was precipitated with TCA at a final concentration of 10 % and kept at 0 C for 30 min. The precipitate was collected by centrifugation at 3000 x g for 20 min and washed three times with cold 80 % ethanol. The DNA was hydrolyzed with pancreatic DNase and snake-venom phosphodiesterase by the procedure of Wu and Kaiser (153). After hydrolysis the products, dAMP, dGMP, dCMP and dTMP,

were separated by paper chromatography. Samples of 10-100 µl were spotted on Whatman # 1 filter paper together with non-radioactive reference nucleotides. The chromatogram was developed with a mixture of isopropanol: concentrated hydrochloric acid: water (170:41:28). The spots containing dTMP and dCMP were cut out and the radioactivity was determined. In all cases less than 2 % of the CPM were recovered at the origin of the chromatogram, indicating that the enzymatic hydrolysis was complete. The ratio of the CPM in dTMP to that in dCMP was determined and corrected for the relative abundance of those nucleotides in the DNA.

In the case of strain tsA13 cdd-1, cells were grown overnight in MMC. The culture was then diluted ten-fold in MMC supplemented with 2 µg/ml of CR and uracil. One hour after dilution radioactive CR or CdR were added to the medium. Cells were harvested for DNA extraction 4 h after the addition of radioactive material (3-4 generations).

12. Labelling of PBS 1 DNA with radioactive bases and nucleosides in various bacterial mutants.

Mutant strains of B.subtilis were grown in PA to a turbidity of 80 Klett units and infected with PBS 1 at an MOI of 4. Two min prior to the addition of radioactive material 2 µg/ml each of unlabelled CR and

uracil were added to the cultures for reasons outlined earlier. Radioactive bases or nucleosides (5-^{3}H-uracil, 5-^{3}H-UdR, 5-^{3}H-CdR or 5-^{3}H-CR) were added at 18 min after infection at which time phage DNA synthesis began (115). Cell lysis was complete after 50-70 min. Cell debris were removed by centrifugation at 7000 x g for 10 min. The labelled phage particles were collected by centrifugation at 40,000 x g for 60 min together with 2 x 10^{10} carrier phage particles. The phage was resuspended in buffer containing 0.02 M Tris-Cl (pH 7.5), 0.1 M sodium chloride and 0.01 M magnesium sulphate and treated with lysozyme (100 µg/ml), DNase (10 µg/ml) and RNase (10 µg/ml) at 37 C for 45 min. The phage was lysed with sodium laurylsulphate (0.45 % final concentration) to release the DNA. Samples were treated twice with an equal volume of cold phenol saturated with 1 x SSC to remove protein. The aqueous layer was separated by centrifugation. Phenol was removed by dialysing twice against 1 x SSC. The dialysate was treated with potassium hydroxide (0.3 N) for 18 h at 37 C to hydrolyse RNA. The phage DNA was precipitated together with 200 µg of unlabelled B.subtilis DNA and hydrolysed as described above.

After hydrolysis the products were separated by paper chromatography as described in the case of bacterial DNA. Samples (10-100 µl) were spotted on Whatman #1 filter paper together with non-radioactive

reference nucleotides. The spots containing dUMP and dCMP were cut out and the radioactivity was determined. The ratio of the CPM in dUMP to that in dCMP was calculated and corrected for the relative abundance of dUMP and dCMP in PBS 1 DNA.

In order to investigate the reproducibility of the method, samples of DNA were hydrolyzed in two portions. Each hydrolysate was chromatographed twice and each chromatogram was counted twice by liquid scintillation counting. The figures in Table 34 were obtained as an average of eight values for each DNA sample. The spread in the values obtained was indicated for each sample when samples were analysed in this way. The largest variations occurred between two chromatograms of the same hydrolysate. It can be seen from Table 34 that the variations were generally less than 10 %, except in some cases that the CPM were low. The results of duplicate experiments never varied by more than 10 %.

13. <u>Chemicals</u>.

Common chemicals were obtained from Fisher Scientific Co. and were all reagent grade. Nitrosoguanidine was obtained from Aldrich Chem. Co. Nucleic acid bases, nucleosides and nucleotides as well as aminopterin, D-L-tetrahydrofolic acid, creatine phosphate, creatine phosphokinase and hydroxyurea were obtained from Sigma Chemical Co.

Dnase, RNase, snake-venom phosphodiesterase, lysozyme and pronase were obtained from Worthington Biochemical Corp.

Rifamycin was a gift of Dr. K.B. Freeman and fluorocytosine, FCR and FCdR were received as gifts from Hoffman LaRoche Chem. Co.

Prepared media were obtained from Difco. Membrane filters were obtained from Schleicher and Schuell (Type B6 Bac-T-Flex; pore size: 0.45 um). Dowex-1-formate (Agl-X8 200-400 mesh) was obtained from Bio-Rad Laboratories. Aquascint II was obtained from International Chemical and Nuclear Corp. Spectrafluor was obtained from Amersham/Searle Corp.

The following radioactive compounds were obtained from Schwarz Mannheim BioResearch Corp.: 5-^{3}H-uracil (18 Ci/mmole), 5-^{3}H-UR (20 Ci/mmole), 5-^{3}H-UdR (18 Ci/mmole), 6-^{3}H-uracil (14.4 Ci/mmole), 6-^{3}H-UdR (20 Ci/mmole), 5-^{3}H-CR (19 Ci/mmole), 5-^{3}H-CdR (25 Ci/mmole), 5-^{3}H-dCTP (20 Ci/mmole), 5-^{3}H-dCDP (18 Ci/mmole), 5-^{3}H-dCMP (22 Ci/mmole), 5-^{3}H-dUMP (15 Ci/mmole), 5-^{3}H-UTP (17.4 Ci/mmole), 5-^{3}H-UDP (17.5 Ci/mmole), 5-^{3}H-CTP (19.7 Ci/mmole), 5-^{3}H-CDP (12.5 Ci/mmole), methyl-^{3}H-TdR (6.7 Ci/mmole), 2-^{14}C-CdR (60 mCi/mmole), 2-^{14}C-CR (29 mCi/mmole) and 2-^{14}C-uracil (30 mCi/mmole).
5-^{3}H-dUDP and 5-^{3}H-dCDP in some cases were prepared by the procedure of Maley <u>et al</u>. (153).

Phage antiserum used was the original antiserum against PBS 1 prepared by Dr. I. Takahashi. The K value was reported to be 1830 (129).

CHAPTER III.

THE METABOLISM OF PYRIMIDINE BASES AND NUCLEOSIDES.

In order to study the metabolism of pyrimidine bases and nucleosides in B.subtilis, a number of mutants requiring pyrimidine compounds for growth were isolated by treating wild-type strain SB19E with nitrosoguanidine. Mutagenized cultures were plated on MMC agar supplemented with uracil and replicated onto MMC agar without uracil. Colonies that failed to grow on MMC agar without uracil were selected. By this technique two mutants, pyr-2 and pyr-3 , were isolated. Strain A26 which was previously isolated by Takahashi was also used in this study. These mutants are presumably blocked in the de novo synthesis of UMP, and therefore they require uracil for growth. Mutants affected in the metabolism of nucleosides were isolated from pyr-2 by nitrosoguanidine mutagenesis. The growth requirements, resistance to fluoro-analogues, and the incorporation of radioactive bases and nucleosides were investigated in these mutant strains to obtain information on the metabolism of uracil, UR,UdR, cytosine,CdR and CR in B.subtilis.

Uracil and uridine.

The pyrimidine requiring strains pyr-2,pyr-3 and

A26 grew well on MMC agar supplemented with uracil (Table 2). Uracil may be taken up and converted to UMP in these mutants. In order to determine whether this conversion of uracil to UMP takes place with or without equilibration with the UR pool, the rates of incorporation of both 5-^{3}H-UR and 5-^{3}H-uracil into TCA-precipitable material were measured at the same concentration (5 µCi/ml) and specific activity (10 Ci/mmole). It was found that the rate of incorporation of radioactive UR was about 25 % of the rate of incorporation of radioactive uracil. This indicates that the conversion of uracil to UMP does not involve UR as an intermediate. Uracil may be converted directly to UMP by UMP pyrophosphorylase.

The pyr mutants of B.subtilis grew well on MMC agar supplemented with UR (Table 2). However, it is not known whether UR was broken down to uracil by UR phosphorylase or whether it was directly converted to UMP by UR kinase.

Deoxyuridine.

Mutants pyr-2, pyr-3 and A26 which are blocked in the de novo synthesis of UMP grew on MMC agar supplemented with UdR (Table 2). No reactions are known in bacteria which oxidize the deoxyribose moiety of deoxyribonucleosides or deoxyribonucleotides to ribose. Thus, the growth of pyr mutants of B.subtilis on UdR may be due to breakdown of UdR to uracil and dR-1-P by UdR phosphorylase.

In E.coli and S.typhimurium, UdR is metabolized by TdR phosphorylase as well as TdR kinase (9, 34, 97). Tomita and Takahashi (138) described the presence of an UdR kinase activity in crude extracts of B.subtilis. In the present study, in addition to UdR kinase activity, UdR phosphorylase activity was found in crude extracts of B.subtilis (Table 3). In order to study the relative importance of these two enzyme activities in the metabolism of UdR, the incorporation of 6-^{3}H-UdR into DNA and total nucleic acids was determined in strains SB19E (wild type) and in 167 thyA thyB, which requires thymine for growth. In the former strain UdR can be metabolized to dUMP, dTMP and dTTP. In the latter strain, which lacks dTMP synthetase, UdR cannot be converted to dTTP. Thus, if 6-^{3}H-UdR is converted to dUMP in appreciable amounts, the radioactivity in DNA as a percentage of the radioactivity incorporated into total nucleic acids is expected to be larger in SB19E than in 167 thyA thyB. In fact, there was no difference in the radioactivity in DNA as a percentage of the radioactivity incorporated into total nucleic acids between strains SB19E and 167 thyA thyB (Table 4). This result indicates that the majority of UdR is metabolized through uracil. More than 93 % of the total CPM incorporated into nucleic acids were recovered in the RNA fraction (Table 4).

Further information on the relative importance of UdR phosphorylase and UdR kinase was obtained by labelling

strain tsA13 cdd-1 pyrG1 with 6-^{3}H-UdR, 6-^{3}H-uracil and 5-^{3}H-uracil. This strain requires CR for growth (Table 2) and is probably blocked in the conversion of UTP to CTP. The radioactivity can only be incorporated into dTMP in the DNA when these radioactive precursors are added to this strain. No radioactivity was expected to be incorporated into DNA when 5-^{3}H-uracil was added. If UdR kinase is important in the metabolism of UdR, the radioactivity in DNA as a percentage of the radioactivity incorporated into total nucleic acids should be higher after labelling with 6-^{3}H-UdR than with 6-^{3}H-uracil. It was found, however, that the radioactivity in DNA as a percentage of the radioactivity incorporated into total nucleic acids was very small with 6-^{3}H-UdR and also with 6-^{3}H-uracil (Table 5). This result indicates that only a very small fraction of the UdR (about 4 %) is metabolized through dUMP. As expected no incorporation into DNA was observed with 5-^{3}H-uracil, indicating that this strain was blocked in the conversion of UTP to CTP.

The UdR kinase activities in extracts of strains SB19E and A26 were 0.38 and 0.48 nmole/h/mg protein, respectively, in the presence of ATP or dATP. When ATP or dATP was omitted from the reaction mixture no UdR kinase activity was detectable. Thus, the activity is completely dependent on the presence of ATP or dATP in the reaction mixture. Labelling experiments described above indicate that most UdR is metabolized through uracil. The activity of UdR

kinase observed *in vitro* therefore appears to be not important in the cells.

Strain *ts*A13 *cdd*-1 and *dns*-2 are affected in the reduction of ribonucleotides and require deoxyribonucleosides for growth (strain *ts*A13 *cdd*-1 requires NdR only at 45 C). Thus these mutants might have higher levels of deoxynucleoside kinase activity. However, the specific activities of UdR kinase in strains *ts*A13 *cdd*-1 and *dns*-2 were not significantly higher than those in strains SB19E and A26. This would be a further indication that UdR kinase does not play a significant role in the metabolism of UdR *in vivo*.

Cytosine.

Mutants *pyr*-2, *pyr*-3 and A26 did not grow on MMC agar supplemented with cytosine at 30 µg/ml (Table 2). In contrast, *pyr* mutants of *E.coli*, or *S.typhimurium* or *S.cerevisiae* are able to grow on cytosine (9, 21, 47, 97). It is not known at present whether the inability of the *B.subtilis* mutants to use cytosine is related to the uptake of this compound or to the conversion of cytosine to other pyrimidine compounds.

A *B.subtilis* mutant carrying a *pyr*G mutation grow on MMC agar supplemented with CR but not on the medium containing cytosine (Table 2). This indicated that cytosine was not converted to CMP in this strain. Thus, CMP

pyrophosphorylase and CR phosphorylase seem to be absent in B.subtilis.

Cytosine deaminase in E.coli and S.typhimurium deaminates fluorocytosine and consequently this base analogue inhibits the growth of these bacteria (9, 21, 97). Since B.subtilis did not seem to be able to metabolize cytosine, fluorocytosine was not expected to inhibit the growth of B.subtilis. However, this compound was found to inhibit growth of B.subtilis as well as of E.coli and S.typhimurium. The inhibition of growth of B.subtilis was not overcome by the addition of TdR to the medium. This would suggest that fluorocytosine did not exert its effect in B.subtilis merely by inhibiting thymidylate synthetase.

If fluorocytosine were deaminated in B.subtilis, strain 3046 (fur-1) would be resistant to both fluorocytosine and flourouracil. However, it was found that the growth of strain 3046 was inhibited by fluorocytosine. This analogue, therefore, may not be deaminated in B.subtilis. An alternative explanation would be that strain 3046 may take up fluorocytosine but not fluorouracil.

Deoxycytidine.

Mutants pyr-2, pyr-3 and A26 grew well on MMC agar supplemented with CdR (Table 2). Therefore, B.subtilis may be able to convert CdR to UdR as in the case of E.coli and S.typhimurium (9, 63, 97).

The metabolism of CdR was further investigated by isolating mutants which were unable to deaminate CdR. A culture of pyr-2 was treated with nitrosoguanidine and plated on MMC agar supplemented with UdR. After an incubation of two days colonies were replicated onto MMC agar supplemented with CdR. A strain that failed to grow was selected and designated as pyr-2 cdd-1. CdR deaminase activity was present in crude extracts of strains SB19E and pyr-2, but absent in extracts of pyr-2 cdd-1 (Table 6).

In contrast to the cases of E.coli and S.typhimurium (9, 97), the cdd-1 mutant of B.subtilis was not resistant to FCdR (Table 2). This result may be explained by the presence of a CdR kinase reported by Bazill and Karamata (8) and Rima and Takahashi (115). Mutants resistant to FCdR were isolated from strain pyr-2 cdd-1 by nitrosoguanidine mutagenesis followed by selection on MMC agar supplemented with uracil and FCdR. Three FCdR resistant strains were investigated further. They incorporated 5-^{3}H-CdR into DNA at rates 1.5 - 4.1 % of the rate in the parent strain (Table 7). Furthermore, these mutants had less than 1 % of the CdR kinase activity of the parent strain (Table 7). These mutants were designated as dck-1, dck-2 and dck-5.

It appears that CdR is metabolized in B.subtilis by two enzymes : CdR deaminase converts CdR to UdR and CdR kinase converts CdR to dCMP. The relative amount of CdR metabolized by those two enzymes has not been established rigorously. Incorporation experiments with 5-^{3}H-CdR in wild

type strains showed that more than 95 % of the total radioactivity incorporated into nucleic acids was incorporated into DNA. This indicates that most CdR in these cells is converted by CdR kinase to dCMP and dCTP and only a small fraction of CdR is converted to UdR and uracil.

Cytidine.

Mutants pyr-2, pyr-3 and A26 grew well on MMC agar supplemented with CR (Table 2). A mutant carrying the cdd-1 mutation was unable to deaminate CdR as well as CR (Table 6) and failed to grow on MMC agar supplemented with CR (Table 2). These results indicate that growth of pyr mutants of B.subtilis on CdR or CR requires a functional deoxycytidine-cytidine deaminase.

Strains of B.subtilis carrying the cdd-1 mutation, were able to incorporate 5-^{3}H-CR into nucleic acids at very high rates (Table 10). This indicates that CR can be converted to CMP, CDP and CTP. In E.coli and S.typhimurium this conversion is mediated by UR kinase (9, 97) and this explains the ability of pyrG mutants to grow on CR. A kinase enzyme specific for CR is absent in the enteric bacteria. In B.subtilis the incorporation of radioactive CR was much more rapid than the incorporation of radioactive UR (Table 10). Thus, kinase specific for CR may be involved in the metabolism of CR in B.subtilis. This question was further investigated by isolating mutants which were unable

to incorporate radioactive CR into mucleic acids. Derivatives of strains tsA13 cdd-1 dck-3 and tsA13 cdd-1 ddd-3 which were resistant to fluorocytidine (FCR) were isolated after nitrosoguanidine mutagenesis. These mutants were selected on MMC agar supplemented with FCR and designated as crk (Table 8). In order to study whether these crk mutants were deficient in CR kinase, the incorporation of 5-^{3}H-CR into nucleic acids was measured. The rate of incorporation of 5-^{3}H-CR in the crk mutants was found to be less than 0.5 % of the rate of the parent strains (Table 9). The activities of CR kinase in crude extracts of the crk mutants and their parent strains confirmed that these mutants were lacking CR kinase (Table 9).

CR kinase was present in tsA13 cdd-1 as well as its derivative tsA13 cdd-1 dck-3 which lacks CdR kinase. Both strains incorporated 5-^{3}H-CR into nucleic acids at about the same rate. This result suggests that CR kinase is distinct from CdR kinase in B.subtilis.

In order to determine whether CR kinase is also distinct from UR kinase in B.subtilis, the incorporation of 5-^{3}H-UR and 5-^{3}H-CR into nucleic acids was measured in a crk mutant and its parent strain. The rate of incorporation of 5-^{3}H-UR into nucleic acids in strain tsA13 cdd-1 dck-3 was very similar to that in tsA13 cdd-1 dck-3 crk-1. On the other hand, the rate of incorporation of 5-^{3}H-CR into tsA13 cdd-1 dck-3 crk-1 was very much lower than that in

*ts*A13 *cdd*-1 *dck*-3 (Table 10). This result indicates that CR kinase and UR kinase are distinct enzymes in *B.subtilis*.

The relative amount of CR metabolized by deamination to UR and that by CR phosphorylation to CMP have not been investigated.

Table 2.

Response of various mutants to pyrimidine compounds.

Strain	Growth on MMC agar supplemented with									
	U	UR	UdR	C	CdR	CR	T	TdR	U+FCdR	none
SB19E (wild type)	+	+	+	+	+	+	+	+	-	+
A26 (*pyr*-1)	+	+	+	-	+	+	-	-	-	-
pyr-2 or *pyr*-3	+	+	+	-	+	+	-	-	-	-
pyr-2 *cdd*-1	+	+	+	-	-	-	-	-	-	-
pyr-2 *cdd*-1 *dck*-1	+	+	+	-	-	-	-	-	+	-
*ts*A13 *cdd*-1 *pyrG*1	-	-	-	-	-	+	-	-	-	-

CR, CdR, cytosine, UR, UdR and uracil were added to MMC agar at a concentration of 30 µg/ml. TdR and thymine were added to a concentration of 20 µg/ml. The concentration of FCdR was 1 µg/ml.

Symbols: +, growth; -, no growth.

Table 3.

UdR kinase and UdR phosphorylase activity in B.subtilis mutants.

Strain	UdR kinase specific activity[a]	UdR phosphorylase specific activity[a]
tsA13 cdd-1	0.23	0.07
SB19E dns-2	0.07	0.41

[a] expressed as nmoles/h/mg protein.

Table 4.

Incorporation of 6-^{3}H-UdR into nucleic acids.

Strain	Time in min	CPM incorporated in total nucleic acids	CPM incorporated into DNA	% of CPM in DNA
SB19E (wild type)	4	2689	156	5.8
	8	8643	521	6.0
	12	18260	1191	6.5
	20	50076	3403	6.8
167 thyA thyB	4	4024	342	8.5
	8	11496	767	6.7
	12	22096	1315	6.0
	20	54835	3361	6.1

Cells of strains SB19E and 167 thyA thyB were grown in PA supplemented with 10 µg/ml thymine. 6-^{3}H-UdR was added to the culture at the late exponential phase at a final concentration of 2.5 µCi/ml. The incorporation was measured in 0.1 ml-samples of the culture in this experiment.

Table 5.

Incorporation of 6-^{3}H-UdR, 6-^{3}H-uracil and 5-^{3}H-uracil into nucleic acids.

Radioactive label	Time in min	CPM incorporated into total nucleic acids	CPM incorporated into DNA	% of CPM in DNA
6-^{3}H-UdR	0	117	66	
	5	15705	616	3.5
	10	41459	2144	5.0
6-^{3}H-uracil	0	156	127	
	5	6057	161	0.6
	10	22324	320	0.9
5-^{3}H-uracil	0	166	58	
	5	3875	59	
	10	13579	63	

Cells of SB19E tsA13 cdd-1 pyrG1 were grown in PA supplemented with 20 µg/ml CR. Radioactive materials were added to the culture at a final concentration of 5 µCi/ml.

Table 6.

Cytidine and deoxycytidine deaminase in B.subtilis.

Strain	CdR deaminase specific activity[a]	CR deaminase specific activity[a]
SB19E (wild type)	2.28	--
pyr-2	1.86	0.55
pyr-2 cdd-1	<0.01	< 0.003

[a] expressed as nmoles/h/mg protein.

Table 7.

Characterisation of CdR kinase mutants.

Strain	CdR kinase specific activity[a]	CdR kinase relative activity (%)	Rate of incorporation of 5-^{3}H-CdR in DNA (%)[b]
pyr-2	3.57	100	--
pyr-2 cdd-1	3.08	86	100[c]
pyr-2 cdd-1 dck-1	0.05	2	1.5
pyr-2 cdd-1 dck-2	0.05	2	4.1
pyr-2 cdd-1 dck-5	0.05	2	1.5

[a] expressed as nmoles/h/mg protein.

[b] Cells of the various mutants were grown in PA supplemented with 10 µg/ml uracil. 5-^{3}H-CdR was added to the cultures at the late exponential phase at final concentrations of 1 µCi/ml. The incorporation of radioactive material into nucleic acids was measured in 0.2 ml samples of the culture at 10 min after addition of the radioactive material.

[c] 10,800 CPM incorporated per 10 min at 0.2 ml culture.

Table 8.

Growth of fluorocytidine resistant strains.

Strain	Concentration of fluorocytidine				
	none	5 μg/ml	25 μg/ml	40 μg/ml	60 μg/ml
SB19E (wild type)	++	+	-	-	-
tsA13 cdd-1	++	+	-	-	-
tsA13 cdd-1 dck-3	++	+	-	-	-
tsA13 cdd-1 dck-3 crk-1	++	+	+	+	+
tsA13 cdd-1 dck-3 crk-2	++	+	+	+	+
tsA13 cdd-1 ddd-3	++	+	±	-	-
tsA13 cdd-1 ddd-3 crk-3	++	+	+	+	+
tsA13 cdd-1 ddd-3 crk-4	++	+	+	+	+

FCR was added to MMC agar at the various concentrations indicated.

Symbols: ++, growth; +, light growth; ± very light growth; -, no growth.

Table 9.

Characterisation of CR kinase mutants.

Strain	Relative rate of incorporation of 5-^{3}H-CR (%)	CMP-CDP-CTP formed per 3 min (pmoles)
tsA13 cdd-1		14.32
tsA13 cdd-1 dck-3	100	14.02
tsA13 cdd-1 dck-3 crk-1	0.1	0.07
tsA13 cdd-1 dck-3 crk-2	0.1	0.17
tsA13 cdd-1 ddd-3	100	9.52.
tsA13 cdd-1 ddd-3 crk-3	0.05	0.09
tsA13 cdd-1 ddd-3 crk-4	0.4	0.59

Cells of the various mutants were grown in PA. 5-^{3}H-CR was added to the culture at the late exponential phase at at final concentration of 6.5 µCi/ml. The incorporation of radioactivity was measured at 5 min after the addition of radioactive material.

Table 10.

Incorporation of 5-^{3}H-UR and 5-^{3}H-CR into nucleic acids.

Radioactive label	Time in min	CPM incorporated in tsA13 cdd-1 dck-3	CPM incorporated in tsA13 cdd-1 dck-3 crk-1
5-^{3}H-UR	0	1445	837
	3	16812	14217
	6	15785	15791
5-^{3}H-CR	0	9604	148
	3	257732	359
	6	331308	515

Cells of both strains were grown in PA to the late exponential phase. Cultures were then mixed with 5-^{3}H-CR or 5-^{3}H-UR at final concentrations of 5 µCi/ml and 20 µCi/ml, respectively.

CHAPTER IV.

METABOLISM OF PYRIMIDINE NUCLEOTIDES.

Ribonucleotides.

The observation that the cdd mutant did not grow on MMC agar supplemented with CR suggested the absence of a CMP, CDP or CTP deaminase in B.subtilis (Table 2). Further evidence for the lack of these enzymes was obtained from the following experiments. The DNA of strain tsA13 cdd-1 ddd-3 which lacks dCDP deaminase and deoxycytidine-cytidine deaminase was labelled with ^{14}C-CR for more than two generations. The DNA was hydrolysed and the ratio of the specific activity of dTMP to that of dCMP was determined [a]. It was found that the ratio $dTMP^{x}/dCMP^{x}$ in strain tsA13 cdd-1 ddd-3 was 0.03 (Table 26), indicating that in this strain no appreciable amounts of CR were converted to uridine ribonucleotides, deoxyribonucleotides and thymidine nucleotides.

The following experiments were carried out to study the conversion of uridine ribonucleotides to cytidine ribonucleotides in B.subtilis. The DNA of strains SB19E and

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[a] Hereafter the ratio of the specific activity of dTMP to that of dCMP will be indicated as $dTMP^{x}/dCMP^{x}$.

tsA13 cdd-1 was labelled with 5-^{3}H-UR or with 5-^{3}H-uracil. It was found that the dCMP in the DNA was labelled (Table 26), indicating that UR and uracil can be converted to UTP, CTP and dCTP. This result is in agreement with results obtained by Barlati (6) which suggest that uracil and uridine can be converted to uridine ribonucleotides and subsequently to cytidine ribonucleotides and deoxyribonucleotides.

Mutants of S.typhimurium which are blocked in the conversion of uridine ribonucleotides to cytidine ribonucleotides require CR for growth (9). Such mutants were also isolated in B.subtilis by treating strain tsA13 cdd-1 with nitrosoguanidine. The mutagenized culture was plated on MMC agar supplemented with CR and replicated onto MMC agar. Strains that failed to grow in the absence of CR were selected and designated as pyrG mutants. When 5-^{3}H-uracil was added to a culture of one such mutant, tsA13 cdd-1 pyrG1, no incorporation of radioactivity into DNA was observed (Table 5). It appears that the conversion of uridine ribonucleotides to cytidine ribonucleotides is blocked in the pyrG1 strain. In all other organisms studied (97), CTP synthetase, which converts UTP to CTP, is responsible for the conversion of uridine ribonucleotides to cytidine ribonucleotides. It is probable that the same enzyme is also involved in B.subtilis.

Deoxyribonucleotides.

Thymidine nucleotides: Wilson *et al*. (150) and Anagnostopoulos and Schneider (2) showed that in *B.subtilis* the requirement for thymine resulted from mutations in two distinct genetic loci (*thy*A and *thy*B). The *thy*A locus is known to code for thymidylate synthetase, but the enzyme coded by the *thy*B locus is unknown (150).

Förster and Holldorf (quoted in 97) proposed that the *thy*B coded pathway may be involved in a direct conversion of deoxycytidine nucleotides to thymidine nucleotides. Therefore, it was first investigated whether deoxycytidine nucleotides could be converted to thymidine nucleotides in *B.subtilis*. The DNA of strain *pyr*-2 *cdd*-1 was labelled with ^{14}C-CdR for more than two generations. The ratio $dTMP^x/dCMP^x$ was found to be 0.43, indicating that CdR, after conversion to deoxycytidine nucleotides, was converted to thymidine nucleotides.

In order to determine whether deoxycytidine nucleotides were first converted to deoxyuridine nucleotides or whether they were directly converted to thymidine nucleotides *via* the *thy*B coded pathway, four types of mutants were isolated with various combinations of *thy*A and *thy*B genotypes. In addition, the *cdd*-1 mutation (CdR deaminase) was introduced into these strains in order to label only deoxyribonucleotides after the addition of radioactive CdR.

The four strains were 167 tsA13 cdd-1 thyA thyB, 167 tsA13 cdd-1 thyA$^+$ thyB, 167 tsA13 cdd-1 thyA thyB$^+$, and SB19E tsA13 cdd-1 thyA$^+$ thyB$^+$. The tsA13 mutation carried by these strains is irrelevant to the present experiments.

Strain 167 tsA13 cdd-1 thyA thyB was derived from 167 tsA13 thyA thyB by transformation with DNA extracted from pyr-2 cdd-1. One of the transformants, which grew on TBB agar supplemented with 10 µg/ml FCdR, was selected and tested for thymine dependence, sensitivity to aminopterin and the presence of CdR-deaminase and dTMP synthetase. Results shown in Table 11 confirm the genotype of this strain, thyA thyB cdd-1.

Strains 167 tsA13 cdd-1 thyA$^+$ thyB and 167 tsA13 cdd-1 thyA thyB$^+$ were derived from 167 tsA13 cdd-1 thyA thyB by transformation with DNA extracted from wild type cells. The transformants which were capable of growing on MMC agar without thymine were selected and tested for sensitivity to aminopterin. The transformants were separated into two classes on the basis of their sensivity to aminopterin; resistant clones (thyA thyB$^+$) and sensitive clones (thyA$^+$ thyB). Since the two loci are unlinked in transformation it is unlikely that thyA$^+$ thyB$^+$ transformants were present among the aminopterin sensitive clones. One strain from each class was selected and tested for thymine dependence, sensitivity to aminopterin and CdR deaminase and dTMP synthetase (Table 11). The genotypes of the two strains tested were confirmed to be

thyA thyB$^+$ cdd-1 (aminopterin resistant) and thyA$^+$ thyB cdd-1 (aminopterin sensitive).

Strain SB19E tsA13 cdd-1 thyA$^+$ thyB$^+$ was derived from SB19E (wild type) by transformation with DNA extracted from a strain with genotype 167 tsA13 cdd-1 thyA thyB$^+$. Transformants which grew on TBB agar supplemented with 10 µg/ml FCdR were selected. Of 41 transformants selected for the cdd-1 mutation, 6 appeared to carry the tsA13 mutation as well. Their genotype was confirmed to be thyA$^+$ thyB$^+$ cdd-1.

The DNA of the above strains was labelled with ^{14}C-CdR for more than two generations. The ratios dTMP^x/dCMP^x were determined in the strains grown in PA (Table 12). The ratio dTMP^x/dCMP^x was also determined in 167 tsA13 cdd-1 thyA$^+$ thyB and 167 tsA13 cdd-1 thyA thyB$^+$ grown in MMC (Table 12). The expected ratios dTMP^x/dCMP^x, assuming that deoxycytidine nucleotides are directly converted to thymidine nucleotides, are also shown in Table 12. It can be seen that the experimental values differ significantly from the expected values. The results therefore do not support the hypothesis that the thyB coded pathway may be involved in a direct conversion of deoxycytidine nucleotides to thymidine nucleotides. There were no significant differences in the ratios between the thyA$^+$ thyB and the thyA thyB$^+$ strains when grown in either PA or MMC. This suggests that deoxycytidine nucleotides are converted to deoxyuridine nucleotides, and subsequently to

thymidine nucleotides.

Lomax and Greenberg (77) devised a very sensitive assay for the synthesis of thymidine nucleotides. This method is based on the replacement of the tritium atom in the 5-position of radioactive dUMP by a methyl group in the synthesis of dTMP. The tritium is released into the medium in the form of water. To demonstrate the presence of a specific enzyme coded by the thyB locus, the release of tritium from 5-^{3}H-dUMP or 5-^{3}H-dUDP was measured in crude extracts of strains thyA thyB cdd-1, thyA thyB^{+} cdd-1 and thyA^{+} thyB cdd-1. In crude extracts of the thyA thyB mutant no tritium was released, indicating the absence of thymidine nucleotide synthesis from the deoxyuridine nucleotides (Table 13). In extracts of strain thyA thyB^{+}, tritium was released from the 5-position of 5-^{3}H-dUDP (Table 13), although thymidylate synthetase coded by the thyA locus was absent in this mutant. As expected a large amount of tritium was released in crude extracts of strain thyA^{+} thyB (Table 13). The large amount of tritium released in extracts of this strain indicates that the normal thymidylate synthetase is active under the assay conditions described. These results demonstrate that an enzyme capable of synthesizing thymidine nucleotides is present in the thyA thyB^{+} strain, while it is absent in the thyA thyB strain. This is the first report of an enzyme activity associated with the thyB locus.

In order to investigate whether the substrate for the thyB coded enzyme is dUMP or dUDP, the release of tritium from 5-^{3}H-dUMP and 5-^{3}H-dUDP was measured in crude extracts of strain 167 tsA13 cdd-1 thyA thyB^{+}. No significant differences were observed in the amount of tritium released from the two substrates. There is, however, the possibility that dUDP is first dephosphorylated to dUMP which is then used by the enzyme as substrate. The stability of the substrates was therefore investigated in the reaction mixture. 5-^{3}H-dUDP or 5-^{3}H-dUMP was added to the assay mixture and after an incubation of 30 seconds the distribution of tritium in UdR, dUMP, dUDP and dUTP was determined by descending paper chromatography with a mixture of isobutyric acid and 2.3 N ammonium hydroxide (66:34 by volume) as solvent. It was found that 5-^{3}H-dUDP added to the assay mixture was quantitatively converted to 5-^{3}H-dUMP within 30 seconds. The reverse reaction e.g. phosphorylation of 5-^{3}H-dUMP to dUDP was not observed and no significant amount of radioactivity was recovered in the dUTP and UdR spots.

dCDP deaminase: The observation that deoxyuridine nucleotides are intermediates in the conversion of deoxycytidine nucleotides to thymidine nucleotides suggests the presence of a deoxycytidine nucleotide deaminase in B.subtilis. The following experiments were designed to demonstrate such a deaminase.

No dCTP deaminase (137, 138) was detectable in extracts of uninfected cells of strain pyr-2 cdd-1 and the four mutants with varying combinations of thyA and thyB genotypes. On the other hand, as reported by Tomita and Takahashi (137, 138), a high dCTP deaminase activity was found in PBS 1-infected cells (1520 nmoles/h/mg protein). Although no significant amount of dCMP deaminase activity was detected in extracts of 167 toA13 cdd-1 thyA thyB (Table 14), a high level of dCDP deaminase was found in B.subtilis (Table 14). A dCDP deaminase has not been reported before in B.subtilis or any other organism.

The activity of dCDP deaminase was stimulated by $MnCl_2$ and the highest activity was obtained at a concentration of 5 mM (Table 15). $MgCl_2$ and $CaCl_2$ stimulated dCDP deaminase to a lesser extent than $MnCl_2$ did. Tris buffer gave higher activities than potassium phosphate buffer at the same pH. The highest activity was obtained in the Tris buffer at pH 7.0. It is, however, possible that the optimal pH may be lower than 7.0. It was found that for each mole of deoxycytidine nucleotide that disappeared from the reaction mixture an equivalent amount of deoxyuridine nucleotide was formed (Table 16).

The activity of dCDP deaminase in crude extracts showed a sigmoidal increase with increasing substrate concentration (Fig. 2). The K_m and V_{max} of dCDP deaminase were graphically determined by extrapolating the linear part of

a double reciprocal plot and found to be 700± 100 μM and 2.5± 0.5 μmoles/h/mg protein respectively. As shown in Table 17 dCDP deaminase was inhibited by dTTP in a competitive fashion.

Attempts were then made to isolate mutants of B.subtilis which lack dCDP deaminase activity. Strain pyr-2 cdd-1 was mutagenized with nitrosoguanidine and FCdR resistant mutants were selected on MMC agar containing uracil (10 μg/ml) and FCdR (1 μg/ml). Two classes of FCdR resistant mutants showing different levels of incorporation of 5-^{3}H-CdR into DNA were found. One class was unable to incorporate 5-^{3}H-CdR into DNA. These were the dck mutants lacking CdR kinase, described earlier. The second class incorporated 5-^{3}H-CdR into DNA at rates 25 to 33 % of the rate in the parent strain. These mutants were found to lack dCDP deaminase (Table 18). They still possessed CdR kinase activity (Table 18) and were designated as ddd mutants. By the same technique another mutant, tsA13 cdd-1 ddd-3 was isolated from tsA13 cdd-1 as parent strain (Table 19).

The enzyme dCDP deaminase may be involved in the synthesis of thymidine nucleotides and, therefore, the level of dCDP deaminase activity may vary between mutants carrying different combinations of thyA and thyB genotypes. However, no significant difference in the level of dCDP deaminase was found between strains carrying various combinations of thyA and thyB genotypes (Table 19).

Conversion of ribonucleotides to deoxyribonucleotides.

The conversion of ribonucleotides to deoxyribonucleotides in bacteria and mammalian cells is mediated by ribonucleotide reductase (97). Some properties of this enzyme in B.subtilis were investigated and compared with those of E.coli ribonucleoside diphosphate reductase.

Reduction of cytidine ribonucleotides : No reductase activity was detectable when ^{3}H-CMP was added as substrate. The activities of ribonucleotide reductase with ^{3}H-CDP and ^{3}H-CTP were found to be 13 and 62 pmoles/h/mg protein respectively in crude extracts of B.subtilis. The rate of product formation was not constant with time and, in fact, after 10 min of incubation at 37 C the reaction stopped. Therefore, the stability of ^{3}H-CDP and ^{3}H-CTP in the reaction mixture were tested by adding these compounds to the reaction mixture. At various times the distribution of radioactivity in CTP, CDP, CMP and CR spots was determined by descending paper chromatography. It was found that CTP was rapidly dephosphorylated to CMP. CDP was also converted to CMP but at a lower rate. No radioactivity was found in the CR spot.

To determine whether CTP or CDP was the real substrate for ribonucleotide reductase, the concentration of CTP and CDP in the assay mixture was compared with the rate of product formation. ^{3}H-CTP was added to the assay mixture and at various times the distribution of radioactivity in

the CTP, CDP, CMP, dCMP, dCDP and dCTP spots was determined by paper chromatography. Results of such an experiment are shown in Fig. 3. The concentration of CTP in the reaction mixture decreased rapidly and correspondingly an increase in the amount of CMP was observed, suggesting that CTP in our reaction mixture was degraded rapidly. Nevertheless, the only deoxyribonucleotide formed was dCTP. This result indicates that CTP may be the substrate for the reduction of cytidine ribonucleotides in B.subtilis. Thereafter CTP was stabilized in the assay mixture by creatine phosphate and creatine phosphokinase.

Reduction of uridine ribonucleotides : The following experiments were carried out to determine the substrate for the reduction of uridine ribonucleotides. The activity of ribonucleotide reductase was measured with ^{3}H-UMP, ^{3}H-UDP and ^{3}H-UTP as substrate. No detectable ribonucleotide reductase activity was observed when ^{3}H-UMP was added as substrate. The activity of reductase with ^{3}H-UDP and ^{3}H-UTP were found to be 7.8 and 7.4 pmoles/h/mg protein respectively. The stability of the radioactive substrate was analysed in a similar fashion as in the case of cytidine ribonucleotides. It was found that ^{3}H-UTP was rapidly converted to UDP and that an equilibrium was established in the assay mixture in which 8 % of the radioactivity was in UTP, about 74 % in UDP and about 18 % in UMP. When ^{3}H-UDP was added an equilibrium was established

with about 86 % of the radioactivity in UDP, 6 % in UTP and about 8 % in UMP. This equilibrium could be shifted by the addition of creatine phosphate and creatine phosphokinase. When ^{3}H-UDP was added together with these compounds, the radioactivity was distributed as follows: 57 % was in UTP, 37 % in UDP and 6 % in UMP. However, it was found that the activity of ribonucleotide reductase was neither inhibited nor stimulated by the shift in the equilibrium. Thus no conclusion could be drawn regarding the substrate in the reduction of uridine ribonucleotides. Attempts were then made to study the product of the reaction with varying amounts of cell extracts. Both dUDP and dUTP were found as products in the assay mixture (Fig. 4). In all cases, the initial rate of dUTP formation was higher than that of dUDP. Presumably the presence of dUDP in the reaction mixture is due to a dUTPase which converts dUTP to dUDP (Tomita and Takahashi, unpublished observations).

<u>Properties of ribonucleotide reductase</u> : It was found that Tris buffer was inhibitory for reduction of both UTP and CTP and consequently assays were performed in potassium phosphate buffer.

As mentioned earlier, the rate of product formation was never linear with time in both CTP reductase and UTP reductase assays. Attempts were made to eliminate this difficulty. The presence of a serine protease in the

extract could play a role in the degradation of the enzyme during incubation. However, the addition of serine protease inhibitor, phenylmethyl sulphonyl fluoride, to the assay mixture did not improve the linearity of the CTP reductase assay. In L.leichmannii the removal of ribosomes from the crude extract by centrifugation at 100,000 x g has been reported to improve the linearity of the CTP reductase assay (43). However, the removal of ribosomes from crude extracts of B.subtilis did not have the same effect. It is also possible that the reaction was not linear because no electron acceptors were present in the assay mixture. Neither the addition of 0.1 mM dithiothreitol nor the addition of NADPH at concentrations of 0.14 or 1.0 µM improved the linearity of UTP reductase or CTP reductase assays. Higher concentrations of NADPH (more than 10 µM) were found to be inhibitory for both cases.

It was found that the CTP reductase activity was stimulated by $MgCl_2$, but $CaCl_2$ had no effect. The highest activity was found with $MgCl_2$ at a concentration of 15 mM. In contrast, UTP reductase was stimulated by $CaCl_2$ and the highest activity was found at a concentration of 1.2 mM. $MgCl_2$ had no effect on the UTP reductase activity. The effect of the pH was tested over the range of pH 6.6 to 8.2 for both CTP reductase and UTP reductase activity. The highest CTP reductase activity was found with a buffer at pH 7.4. However, the differences in activity over the pH

range tested were very small for CTP reductase activity (less than 14 %) and there was practically no difference in the case of UTP reductase.

Requirement for ATP and the effect of hydroxyurea : The activity of E.coli ribonucleoside diphosphate reductase is dependent on ATP in the assay mixture (72, 113). It was found that the activity of CTP reductase in B.subtilis was inhibited by ATP (Table 20). It was also found that ATP at a concentration of 1.3 mM reduced the UTP reductase activity to 43 % of the control value.

The E.coli ribonucleotide reductase is inhibited in vitro and in vivo by hydroxyurea (33, 122). In B.subtilis, CTP reductase activity was not inhibited by this drug in vitro (Table 20). Similarly, UTP reductase in vitro was not sensitive to hydroxyurea. These results indicate that ribonucleotide reductase in B.subtilis, like in the case of L.leichmannii, (33), is not sensitive to hydroxyurea in vitro.

Results shown in Table 21 indicated that the growth of B.subtilis was inhibited by hydroxyurea. The addition of deoxyribonucleosides to the medium removed the inhibitory effect of hydroxyurea (Table 21). This effect was observed only in strains which were capable of converting the deoxyribonucleosides to deoxyribonucleotides. The dck mutant which lacks CdR kinase was not able to grow on MMC agar containing hydroxyurea and deoxyribonucleosides.

Ribonucleotide reductase in mutant strains : Bazill and Karamata (8) have isolated 22 tsA mutants of B.subtilis which are temperature-sensitive for DNA synthesis. Although the tsA mutants can not grow at 45 C, they do so in the presence of deoxyribonucleosides in the medium. Bazill and Karamata (8) suggest that the tsA mutations may be located in the structural gene for ribonucleotide reductase, since the temperature sensitive phenotype of all 22 tsA mutants can be reversed by deoxyribonucleosides and since the different mutants are reversed by different combinations of deoxyribonucleosides. In order to determine whether the ribonucleotide reductase in the tsA mutants was affected, CTP reductase and UTP reductase activity were assayed in strains 167 tsA13 thyA thyB and in 167 ts^+ thyA thyB. It was found that there was no difference in the activities of CTP reductase or UTP reductase between the ts^+ strain and the tsA13 strain grown in the presence of deoxyribonucleosides at either 30 C or at 45 C. Thus the presence of the tsA13 mutation did not affect the activities of UTP and CTP reductase in the cells.

In order to compare the temperature sensitivity in vitro of the wild type enzyme and the mutant enzyme, both the ts^+ strain and the tsA13 strain were grown at 37 C and CTP reductase activity and UTP reductase activity were assayed at 37 C and at 45 C. The activity of UTP reductase

in the ts$^+$ strain assayed at 45 C was 54 % of the activity assayed at 37 C. In the tsA13 strain, the activity assayed at 45 C was 59 % of that at 37 C. Therefore there seems to be no significant difference in the enzyme in vitro between the wild type strain and mutant strain.

B.subtilis can use exogenous deoxyribonucleosides (8,115) and thus it may be possible to isolate mutants which have an absolute requirement for deoxyribonucleosides (dns). In order to isolate such mutants, strains A26 and SB19E were mutagenized with nitrosoguanidine. The mutagenized cultures were plated on MMC agar supplemented with deoxynucleosides (20 µg/ml) and uracil (10 µg/ml). Subsequently, the colonies were replicated onto MMC agar without deoxyribonucleosides and those which failed to grow in the absence of deoxyribonucleosides were selected and examined further. All mutants isolated by this technique required deoxyribonucleosides for growth at 45 C (Table 22). However, when CTP reductase activity was assayed in the mutant strains it was found that the tsA13 mutant as well as the dns mutants contained appreciable amounts of CTP reductase activity (Table 23). Strains A26 dns-1 and dns-2 required deoxyribonucleosides at 45 C but not at 25 C (Table 24). Strains A26 dns-1 and dns-2 as well as dns-3 were isolated at 37 C. The other dns mutants were isolated at 30 C after this temperature dependency had been noticed. A similar temperature dependency for the requirement for deoxyribonucleosides has been observed by Fuchs and Neuhard (41) in a mutant of E.coli which is affected in ribonucleo-

tide reductase. The temperature dependency of their strain is related to growth rate rather than to higher temperature, since the mutant phenotype can be reversed by slowing the rate of growth of this strain.

Further evidence for the complex nature of the requirement for deoxyribonucleotides was obtained from studies on the effect of other mutations on the tsA phenotype. The growth requirements of a number of strains carrying the tsA13 mutation in combination with other mutations which affect the metabolism of deoxycytidine nucleotides, such as ddd (dCDP deaminase) , cdd (deoxycytidine-cytidine deaminase), thyA and thyB were tested. It was found that strains carrying the tsA13 mutation in combination with the thyB and cdd-1 mutations or with the ddd-3 and cdd-1 mutations were able to grow at 45 C without deoxyribonucleosides (Table 25). It is not known whether a strain with genotype tsA13 ddd-3 cdd^+ can grow at 45 C since mutants of this type are not available.

Synthesis of dTTP.

dTTP may be synthesized in B.subtilis by the following four pathways. Exogenous TdR can be converted to dTMP and to dTTP. UTP may be reduced to dUTP which can be converted to dUMP → dTMP → dTTP. CTP may be reduced to dCTP which can be converted to dCDP → dUDP → dUMP → dTMP → dTTP. Exogenous CdR can be converted to dCMP and dCDP which

is deaminated to dUDP → dUMP → dTMP → dTTP.

In order to evaluate the contributions of these pathways, the DNA of various bacterial mutants was labelled for more than two generations with radioactive bases or nucleosides. The DNA was then extracted and hydrolysed and the ratio $dTMP^{*}/dCMP^{*}$ was determined. If one can assume that the dCTP and dTTP pools turnover rapidly and that steady-state conditions exist for the synthesis of deoxyribonucleotides, the above ratio $dTMP^{*}/dCMP^{*}$ should determine the contribution of various pathways to the dTTP pool. These assumptions were shown to be valid in E.coli by Neuhard and Thomassen (92). These authors showed that the dCTP pool in E.coli turned over with a half time of 1.1 minute at 30 C. The turnover rate at 37 C was too rapid to be determined by their techniques. Steady-state conditions prevailed for the synthesis of deoxyribonucleotides in a growing culture of E.coli (92).

The results of labelling experiments in which various mutants were labelled with different radioactive bases and nucleosides are shown in Table 26. Control experiments were carried out with strain tsA13 cdd-1 ddd-3 which was defective in dCDP deaminase and deoxycytidine-cytidine deaminase. The addition of either ^{14}C-CdR or ^{14}C-CR to this strain should result in the incorporation of radioactivity into DNA in the dCMP fraction but not in dTMP since no pathways exist in this strain for the conversion of cytidine nucleotides to dTTP. Indeed the DNA of this strain labelled with

^{14}C-CdR and ^{14}C-CR showed a ratio dTMP^{x}/dCMP^{x} of 0.01 and 0.03 respectively, indicating that no dTMP was labelled (Table 26).

The tritium atom in the 5-position of labelled deoxynucleotides is replaced by a methyl group in the synthesis of thymidine nucleotides. When 5-^{3}H-UR was added to wild type cells, the ratio dTMP^{x}/dCMP^{x} was 0.01, indicating that no dTMP was labelled (Table 26).

In order to investigate the contributions of the CTP reductase and CdR kinase pathways in combination with the dCDP deaminase pathway, DNA of strain tsA13 cdd-1 was labelled with ^{14}C-CR or with ^{14}C-CdR in PA and MMC. A significant difference in the ratio dTMP^{x}/dCMP^{x} was observed between DNAs labelled with ^{14}C-CR and with ^{14}C-CdR. This difference was observed in both PA and MMC. This result indicates that labelled nucleotides are exchanged between the dCTP and dCDP pools and that the ratio dCTP^{x}/dCDP^{x} is dependent on the type of label used. When ^{14}C-CdR is used, the ratio dCDP^{x}/dCTP^{x} is greater than 1.0, since dCDP is labelled directly and since the specific activity of the dCTP pool is lowered by cold dCTP derived from the reduction of CTP. (Fig. 5). On the other hand, when ^{14}C-CR is used, the ratio dCDP^{x}/dCTP^{x} is smaller than 1.0, since the dCTP pool is labelled directly and since the specific activity of the dCDP pool is lowered by cold dCDP derived from exogenous CdR (Fig. 5).

The ratio $dTMP^x/dCMP^x$ was 0.19 when the DNA of tsA13 cdd-1 was labelled with ^{14}C-CR in PA (Table 26). This indicates that 19 % of the dTTP in these cells in PA is derived from dCTP. The ratio $dTMP^x/dCMP^x$ in DNA of tsA13 cdd-1 labelled in MMC with ^{14}C-CR was 0.39 (Table 26). This indicates that in MMC 39 % of the dTTP is derived from the reduction of CTP and the remainder from UTP. The difference in the percentages of dTTP derived from the dCTP between PA and MMC (19 % and 39 % respectively) may be due to the presence of exogenous CdR in PA which can be converted to dTTP by the following path: dCMP $\longrightarrow$ dCDP $\longrightarrow$ dUDP $\longrightarrow$ dUMP $\longrightarrow$ dTMP $\longrightarrow$ dTTP.

The contribution of exogenous TdR to the synthesis of dTTP was determined by labelling the DNA of strain tsA13 cdd-1 dck-3 crk-1 with ^{14}C-uracil. In this strain which lacks CdR-CR deaminase, CdR kinase and CR kinase, the specific activity of dCTP must be the same as that of dUMP since the specific radioactivity of the nucleotide pools can not be diluted by exogenous bases and nucleosides in this strain. Exogenous TdR, however, can dilute the radioactivity of the thymidine nucleotides in this strain and the amount of dilution is a measure of the participation of exogenous TdR in the synthesis of dTTP. The DNA of the above mentioned strain was labelled with ^{14}C-uracil and the ratio $dTMP^x/dCMP^x$ was determined. The ratio was found to

be 0.83 (Table 26), indicating that in this strain in PA about 17 % of dTTP was derived from exogenous TdR and the remainder from the reduction of UTP and CTP.

Table 11.

Properties of mutants carrying thyA and thyB mutations.

Genotypes of strains used [a]	Thymine dependence	Growth on MMC + T + aminopterin [b]	dTMP-synthetase specific activity [c]	CdR deaminase specific activity [c]
thyA thyB cdd-1	+	+	not detected	<0.01
thyA$^+$ thyB cdd-1	-	-	19	<0.01
thyA thyB$^+$ cdd-1	-	+	not detected	<0.01
thyA$^+$ thyB$^+$ cdd-1	-	-	13	<0.01

a all these strains carry the tsA13 mutation in addition to the mutations indicated.

b thymine and aminopterin were added to final concentrations of 10 µg/ml and 200 µg/ml respectively.

c expressed as nmoles/h/ mg protein.

Table 12.

Ratios of $dTMP^{*}$ to $dCMP^{*}$ in various mutants labelled with ^{14}C-CdR.[a]

Genotypes of strains used[b]	Cells grown in PA		Cells grown in MMC	
	$dTMP^{*}/dCMP^{*}$ observed	$dTMP^{*}/dCMP^{*}$ expected[c]	$dTMP^{*}/dCMP^{*}$ observed	$dTMP^{*}/dCMP^{*}$ expected[c]
thyA thyB cdd-1	0.02	0.0	not determined	0.0
thyA^{+} thyB cdd-1	0.22	0.0	0.43	0.0
thyA thyB^{+} cdd-1	0.23	$\geqslant$ 0.0	0.52	$\geqslant$ 1.0
thyA^{+} thyB^{+} cdd-1	0.37	0.4[d]	not determined	$\geqslant$ 0.4[d]

a ^{14}C-CdR was added to the cultures at a final concentration of 0.1 μCi/ml.

b all these strain carry the tsA13 mutation in addition to the ones indicated.

c assuming that the thyB coded pathway involves a direct conversion of deoxycytidine to thymidine nucleotides.

d based on the ratio of $dTMP^{*}$ / $dCMP^{*}$ in strain pyr-2 cdd-1.

Table 13.

Activity of the thyB coded thymidylate synthetase in various mutants.

Genotype of strains used[a]	CPM in assay supernatant fluid[b]	CPM in control supernatant fluid[b]
thyA thyB cdd-1	1627	1698
thyA thyB$^+$ cdd-1	36694	9228
thyA$^+$ thyB cdd-1	418868	4483

5-^{3}H-dUDP was used as substrate in this experiment.

[a] These strains carry the tsA13 mutation in addition to the ones indicated.

[b] CPM in 0.5 ml of supernatant fluid. The CPM in the control were those released into the medium without incubation at 37 C.

Table 14.

dCMP deaminase and dCDP deaminase activities in mutant thyA thyB$^+$cdd-1.

Substrate added to the assay mixture [a]	Deaminase activity (nmoles/h/ mg protein)
5-^{3}H-dCDP	not detectable
5-^{3}H-dCDP + 167 μM dCDP	≈ 41
5-^{3}H-dCDP + 167 μM dCMP	not detectable
5-^{3}H-dCMP	not detectable
5-^{3}H-dCMP + 167 μM dCMP	< 1.5

[a] the assay mixture consisted of substrate, 25 mM Tris-Cl buffer pH 7.5, 125 μM $MgCl_2$ and aliquots of crude extract corresponding to 1.95 mg protein in a total volume of 0.6 ml.

Table 15.

Effect of divalent cations on dCDP deaminase activity.

Cations	Concentration (mM)	Relative activity (%) [a]
$MnCl_2$	1.25	87
	5.0	100
	7.5	89
	15	83
$MgCl_2$	1.25	38
	5.0	42
	10	41
	20	21
$CaCl_2$	1.25	19
none	--	14

[a] 100 % activity was 113 nmoles/h/ mg protein. This assay was not performed under optimal conditions of substrate concentration and pH.

Table 16.

The stoichiometry of the dCDP deaminase reaction.

Incubation time	µmoles in the assay mixture				Total µmoles	Total µmoles	Total µmoles
	dCMP	dCDP	dUMP	dUDP	dCMP + dCDP	dUMP + dUDP	recovered
boiled extract	0.57	0.47	0.00	0.00	1.04	0.00	1.04
10 min	0.35	0.19	0.27	0.15	0.54	0.42	0.96
20 min	0.35	0.14	0.34	0.19	0.49	0.53	1.02

The conditions of this experiment are as described in Materials and Methods.

Table 17.

The effect of dTTP on the activity of dCDP deaminase.

Concentration of dCDP (μM)	Concentration of dTTP (μM)	dCDP deaminase activity [a]	Inhibition (%)
334	none	806	0
	45	758	6
	90	709	12
	180	693	14
	360	669	17
	720	443	45
100	none	304	0
	360	164	46
	720	52	83

[a] expressed as nmoles/h/ mg protein.

Table 18.

dCDP deaminase and CdR kinase in ddd mutants.

Strain	dCDP deaminase specific activity[a]	CdR kinase specific activity[a]
pyr-2 cdd-1	10	3.33
pyr-2 cdd-1 dck-1	17	<0.01
pyr-2 cdd-1 ddd-1	<0.5	2.76
pyr-2 cdd-1 ddd-2	<0.5	not determined

[a] expressed as nmoles/h/ mg protein.

Table 19.

dCDP deaminase activity in mutants affected in thymidylate synthesis.

Genotypes of strains used [a]	dCDP deaminase specific activity [b]
thyA thyB cdd-1	286
thyA thyB$^+$ cdd-1	224
thyA$^+$ thyB cdd-1	401
thyA$^+$ thyB$^+$ cdd-1	294
tsA13 cdd-1 ddd-3	< 5

[a] all those strains carry the tsA13 mutation in addition to the ones indicated.

[b] expressed as nmoles/h/ mg protein.

Table 20.

Effect of ATP and hydroxyurea on CTP reductase activity.

	Concentration (mM)	CTP reductase specific activity[a]	Inhibition (%)
control	--	0.47	0
ATP	0.125	0.29	39
	1.25	0.11	76
hydroxyurea	1	0.43	8
	10	0.47	0

[a] expressed as nmoles/h/ mg protein.

Table 21.

Effect of hydroxyurea on the growth of B.subtilis.

Concentration of hydroxyurea (mM)	NdR[a]	Growth on MMC agar		
		wild type	tsA13 cdd-1	tsA13 cdd-1 dck-3
0	-	++	++	++
1	-	++	++	++
3	-	++	++	++
10	-	++	+	±
30	-	±	-	-
60	-	-	-	-
100	-	-	-	-
60	+	+	+	-
100	+	+	+	-

[a] NdR (AdR + GdR + CdR + TdR) were added at a final concentration of 10 μg/ml each.

Symbols: ++, growth; +, light growth; ±, very light growth; -, no growth.

Table 22.

Growth requirements of dns and tsA13 mutants at 30 C and at 45 C.

Strain	Incubation at 30 C		Incubation at 45 C	
	MMC	MMC+NdR [a]	MMC	MMC+NdR [a]
SB19E wild type	++	++	++	++
tsA13 cdd-1	++	++	-	++
tsA13 cdd-1 dck-3	++	++	-	-
A26 [b]	++	++	++	++
A26 dns-1 [b]	-	+	-	+
dns-2	+	++	-	+
dns-3	+	++	±	+
dns-4	-	++	-	+
dns-5	-	++	-	+
dns-6	-	++	-	+
dns-7	-	+	-	+
dns-8	-	++	-	+

[a] NdR were added at a final concentration of 20 µg/ml.

[b] uracil was added to the plates on which these strains were tested.

Symbols: ++, growth; +, light growth; ±, very light growth; -, no growth.

Table 23.

CTP reductase activity in dns and tsA13 mutants.

Strain	CTP reductase specific activity[a]	CTP reductase activity (% of parent strain)
SB19E wild type	1.26	100
dns-2	0.77	61
dns-3	0.29	23
dns-4	1.24	98
dns-5	0.88	69
dns-6	0.49	39
dns-7	0.46	36
dns-8	1.14	92
tsA13 cdd-1	1.06	85
A26	1.61	100
A26 dns-1	0.76	44

[a] expressed as nmoles/h/ mg protein.

Table 24.

Temperature sensitivity of dns and tsA13 mutants.

Incubation temperature	A26 dns-1 +NdR[a]	A26 dns-1 -NdR[a]	dns-2 +NdR	dns-2 -NdR	tsA13 cdd-1 +NdR	tsA13 cdd-1 -NdR
22 C	++	±	++	++	++	++
25 C	+	±	++	++	++	++
30 C	+	-	++	+	++	++
37 C	+	-	++	-	++	+
45 C	+	-	++	-	++	-

[a] uracil was also added to the medium for this strain

Symbols: ++, growth; +, light growth; ±, very light growth; -, no growth.

Table 25.

Phenotypic reversion of the tsA13 mutation.

Strain	MMC at 37 C	MMC at 45 C	MMC + NdR at 45 C[a]
167 tsA13 thyA thyB [b]	++	-	+
167 tsA13 thyA thyB$^+$	++	-	+
167 tsA13 thyA$^+$ thyB	++	-	+
167 tsA13 thyA thyB cdd-1[b]	++	+	+
167 tsA13 thyA$^+$ thyB cdd-1	++	+	+
167 tsA13 thyA thyB$^+$ cdd-1	++	-	+
tsA13 cdd-1	++	-	+
tsA13 cdd-1 ddd-3	++	+	+

[a] NdR were added to a final concentration of 10 µg/ml each.

[b] thymine was added to the plates for these strains at a concentration of 10 µg/ml.

Symbols: ++, growth; +, light growth; -, no growth.

Table 26.

The ratios of $dTMP^{x}$ to $dCMP^{x}$ in DNA of various mutants.

Strain	Medium	Type of label	Label µCi/ml	CPM dTMP/dCMP	Ratio $dTMP^{x}/dCMP^{x}$
tsA13 cdd-1 ddd-3	PA	^{14}C-CR	0.1	109/2711	0.03
tsA13 cdd-1 ddd-3	PA	^{14}C-CdR	0.1	48/4440	0.01
SB19E (Wild type)	PA	5-^{3}H-UR	25	5/540	0.01
tsA13 cdd-1	PA	^{14}C-CR	0.1	2780/10800	0.19
tsA13 cdd-1	PA	^{14}C-CdR	0.1	16406/19790	0.62
tsA13 cdd-1	MMC	^{14}C-CR	0.1	1078/2066	0.39
tsA13 cdd-1	MMC	^{14}C-CdR	0.1	4093/4815	0.64
tsA13 cdd-1 dck-3 crk-1	PA	^{14}C-Ura	0.8	9773/8804	0.83

Figure 2.

Lineweaver-Burke plot of the velocity of the dCDP deaminase versus the substrate concentration.

The assay of dCDP deaminase was performed under the standard condition. The amount of product formed was determined after 7.5 min incubation at 37 C.

1/v is expressed as hour/nmole.

1/S is expressed as mM^{-1}.

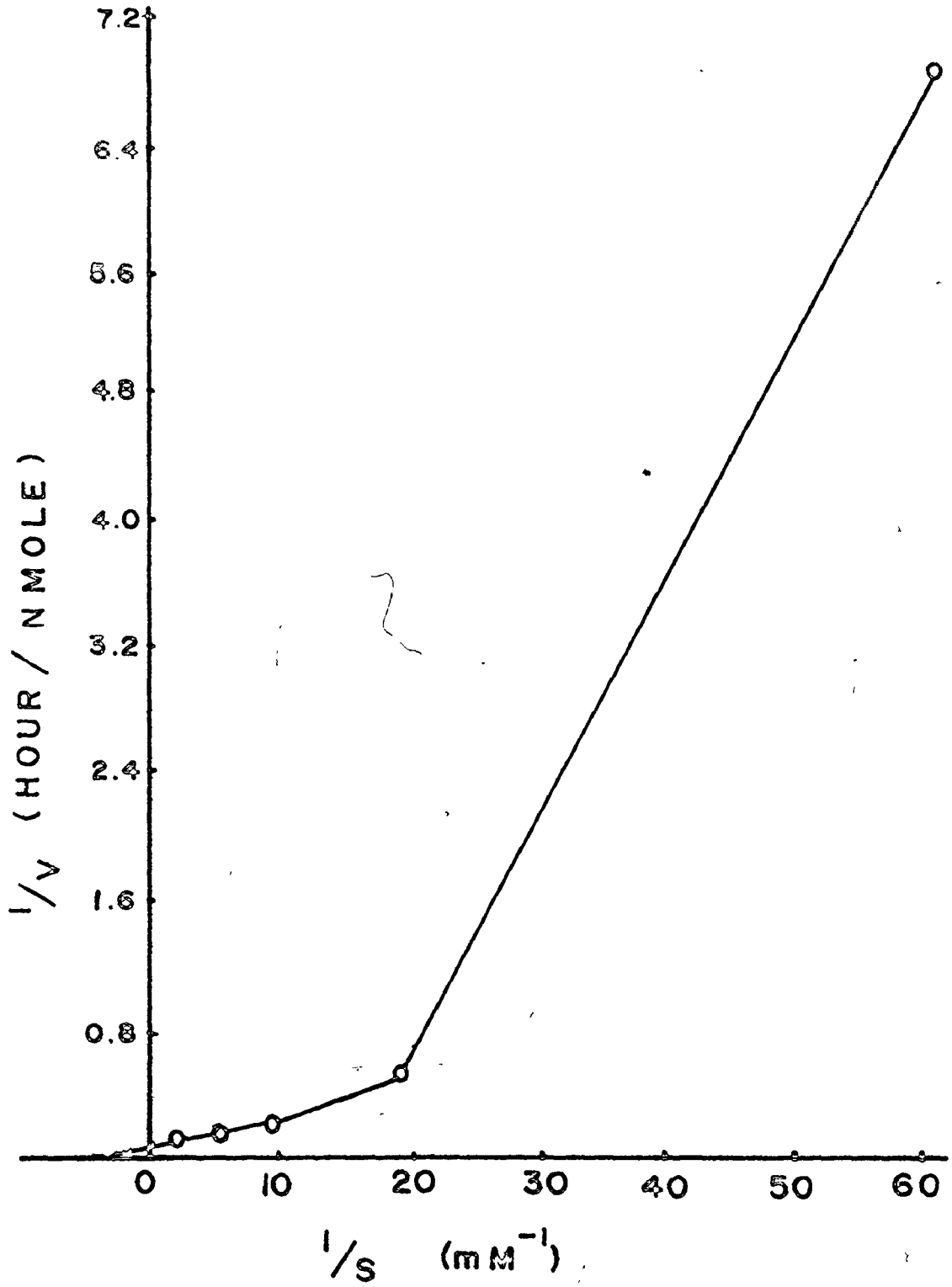

7.2
6.4
5.6
4.8
4.0
3.2
2.4
1.6
0.8
1/V (HOUR / NMOLE)
0
10
20
30
40
50
60
1/S (mM⁻¹)

Figure 3.

Changes in the ribonucleotide and deoxyribonucleotide composition in the assay mixture for CTP reductase.

CTP reductase was assayed under the standard condition, except that no creatine phosphate and creatine phosphokinase were added. Nucleotides and deoxynucleotides were separated by paper chromatography. The % of the total cpm recovered in each fraction was plotted versus the time of incubation at 37 C.

□—□ , CMP

○—○ , CDP

△—△ , CTP

△—△ , dCTP

○—○ , dCDP

□—□ , dCMP

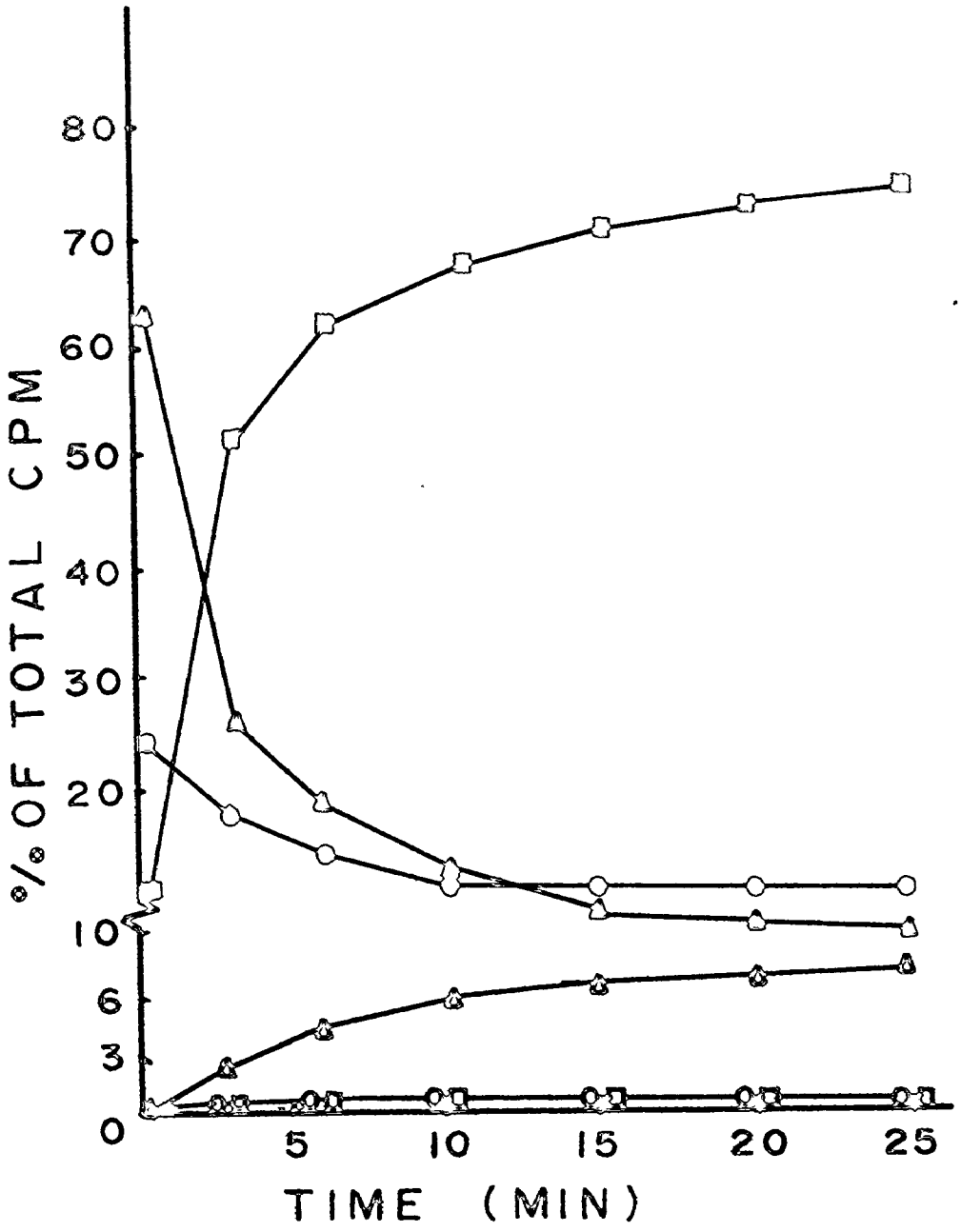

80
70
60
50
40
30
20
10
6
3
0
% OF TOTAL CPM
5
10
15
20
25
TIME (MIN)

Figure 4.

The composition of the products in UTP reductase assays at various times.

UTP reductase assays were performed under the standard condition with varying amounts of crude extract. The products were separated by paper chromatography. The amounts of dUTP, dUDP and dUMP and the total amount of products were plotted versus time.

A: protein concentration was 7.0 mg/ml.

B: protein concentration was 3.5 mg/ml.

C: protein concentration was 1.75 mg/ml.

D: protein concentration was 0.88 mg/ml.

Symbols:

○—○ , total product formed

△—△ , dUTP

○—○ , dUDP

□—□ , dUMP

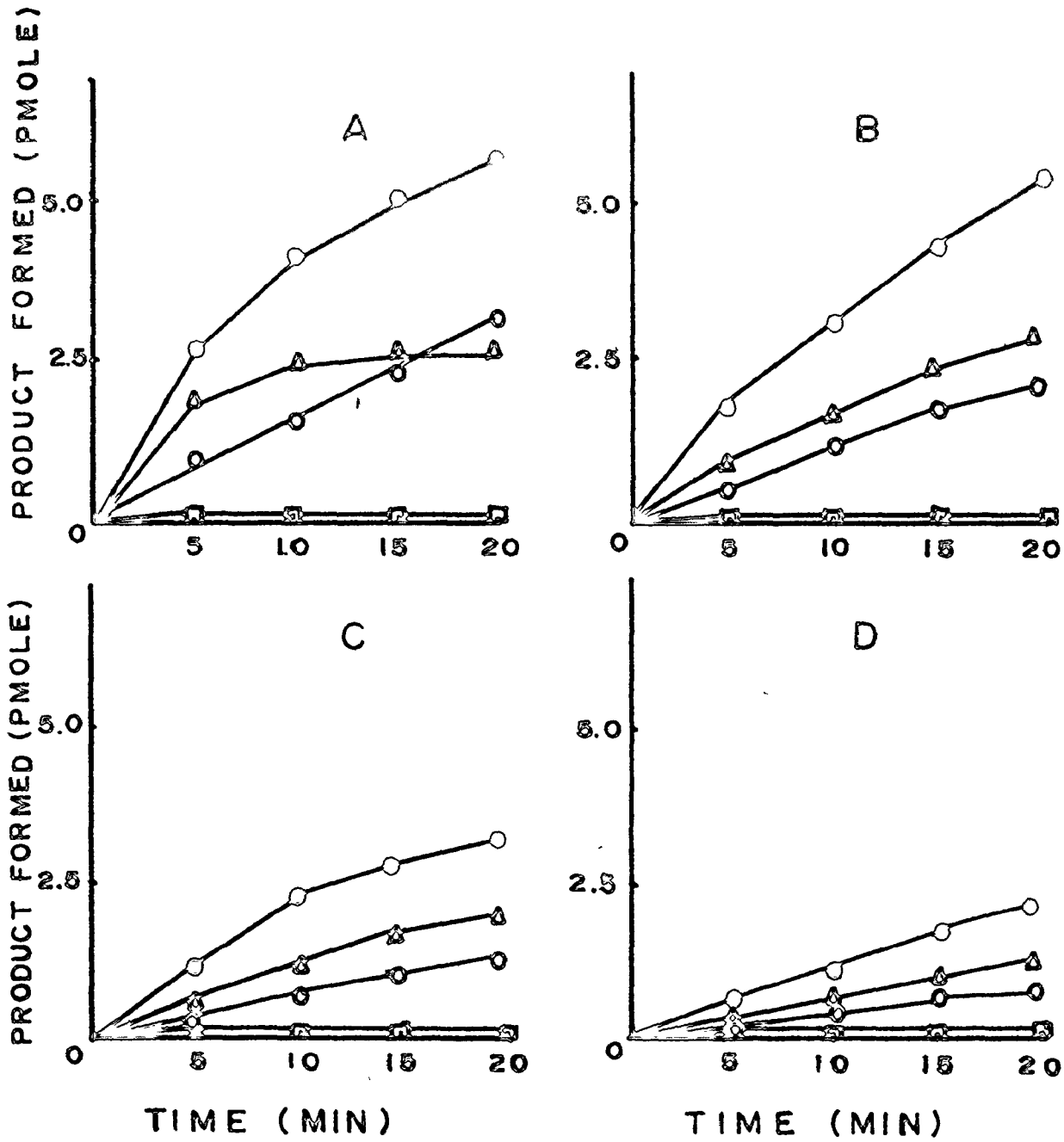
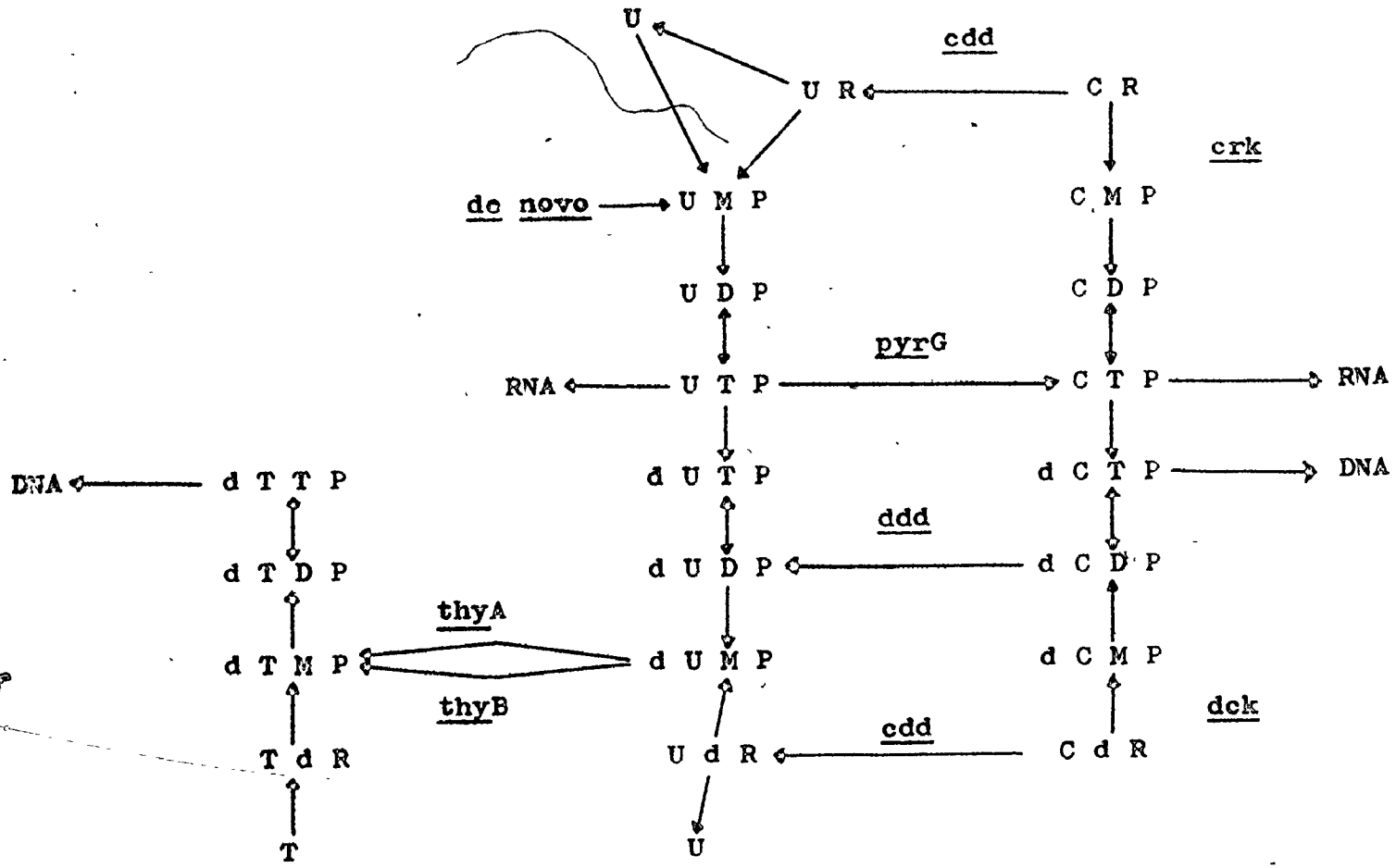

A
B
C
D
PRODUCT FORMED (PMOLE)
5.0
2.5
0
5
10
15
20
TIME (MIN)

Figure 5: METABOLISM OF PYRIMIDINE IN B.SUBTILIS



CHAPTER V.

PYRIMIDINE METABOLISM IN PBS 1-INFECTED CELLS.

Development of the phage.

The process of infection with PBS 1 used in this study was characterised earlier by Takahashi (129). The latent period of the phage was found to be 35 min and the rise period 15 min (129). The burst size of PBS 1 under these conditions was found to be 23 (129).

Number of phage producing cells : For biochemical studies on phage development it is important to know the fraction of the cells that are producing progeny phages. The numbers of phage producing, phage carrying and uninfected cells were determined in cultures of SB19E infected with PBS 1 at an MOI of 5 in either MMC or in PA. In MMC it was found that more than 80 % of the cells were killed by PBS 1 without producing phages and only 4 % of the cells produced progeny phages in MMC (Table 27). These observations led to the conclusion that MMC is not a suitable medium for biochemical studies on the development of PBS 1. In PA, however, 85 % of the cells produced phages and the remaining cells were either uninfected or phage carrying (Table 27). The fraction

of "killed" cells was very small in PA. From the total number of PFU after lysis of the culture and the number of phage producing cells present, it was estimated that each phage producing cell released about 40 PFU after lysis under our conditions.

Nucleic acid metabolism : In order to obtain information on biochemical changes occurring during the development of PBS 1, the rate of RNA and DNA synthesis in infected cells was determined. Cells infected with PBS 1 at an MOI of 5 in PA were pulse-labelled with 5-^{3}H-UR and the incorporation into DNA and RNA was determined.

The apparent rate of RNA synthesis after infection increased gradually and reached a maximal value at 25 min after infection (Fig. 6). The rate of incorporation of 5-^{3}H-UR into DNA began to rise 15 minutes after phage infection and reached a maximal value 30 min after infection (Fig. 6).

The following experiments were conducted to determine host DNA synthesis after PBS 1 infection. The incorporation of methyl-^{3}H-TdR into DNA was measured in SB19E infected with PBS 1. Radioactive TdR can only be incorporated into host DNA and not into phage DNA. As shown in Fig. 7 the synthesis of host DNA was greatly reduced by PBS 1 infection. In a separate experiment, it was found that the rate of incorporation of radioactive TdR into host DNA was about 20 % of the rate of uninfected cells. This

residual host DNA synthesis may be due to the presence of uninfected and phage carrying cells in the culture. The above results appear to indicate that the synthesis of host DNA is shut off soon after infection. However, an alternative explanation for this result may be that radioactive TdR can no longer enter the nucleotide pools in phage-infected cells. Therefore, the rate of DNA synthesis was measured also with 5-^{3}H-CdR. The rate of incorporation of 5-^{3}H-CdR 5 min after infection was again 20 % of the rate of uninfected cells.

It was reported earlier that the development of PBS 1 was completely resistant to rifamycin, an inhibitor of RNA synthesis (15). In the present study, it was found that rifamycin inhibited DNA synthesis in PBS 1-infected cells only slightly, but DNA synthesis in uninfected cells was reduced greatly by the antibiotic (Table 28). When similar experiments were carried out with DNA negative phage mutants, it was found that the rate of DNA synthesis, which was about 20 % of the rate in cells infected with wild type phage, was reduced to the background level by the addition of rifamycin (Table 28). These results may indicate that residual amounts of DNA synthesis in PBS 1-infected cells may be due to the presence of uninfected cells and phage-carrying cells.

<u>Degradation of host DNA</u> : In <u>E.coli</u> infected with phage T4 host DNA is rapidly degraded and deoxyribonucleotides

released are reutilized in the synthesis of phage DNA (144). However, in the PBS 1 system, Tomita (137) found that host DNA was not degraded to acid soluble nucleotides in cells in Tris medium infected at very late exponential phage. In order to investigate host DNA degradation during PBS 1 infection under our conditions, the release of radioactivity from host cells prelabelled with methyl-^{3}H-TdR was measured. No changes occurred in the amount of TCA precipitable radioactivity in the cells infected with PBS 1 at an MOI of 5 in PA (Fig. 8), indicating the absence of host DNA degradation. The apparent absence of host DNA degradation is not due to reutilization of degradation products in phage DNA synthesis, since no enzyme which demethylates dTMP in B.subtilis cells is known.

Pyrimidine metabolism.

The pathways for the metabolism of pyrimidine compounds in uninfected cells of B.subtilis are summarized in Fig. 5. The following experiments were carried out to investigate UdR kinase, CdR deaminase and CdR kinase in PBS 1-infected cells. In addition, changes in dCDP deaminase, ribonucleotide reductase and the thyB coded enzyme were also studied in PBS 1-infected cells. All bacterial mutants used in this study lysed after 60 min after infection indicating that the development of PBS 1 in these strains may be comparable to that in wild type cells.

UdR kinase : In order to investigate induction of UdR kinase, phage infected cells of strain tsA13 cdd-1 pyrG1 dck-4 were labelled with 6-^{3}H-UdR or 6-^{3}H-uracil. A similar experiment carried out with uninfected cells showed that no significant amount of UdR was metabolized by UdR kinase in the uninfected cell and that nearly all UdR was broken down to uracil and deoxyribose-1-phosphate (Table 5). In pyrG strains radioactivity from 6-^{3}H-UdR and 6-^{3}H-uracil can be incorporated only into the dUMP moiety of PBS 1 DNA and not into the dCMP moiety. If UdR kinase were induced by PBS 1, the radioactivity in DNA as a percentage of the radioactivity incorporated into total nucleic acids should be substantially higher after the addition of 6-^{3}H-UdR to the cells than after the addition of 6-^{3}H-uracil, since UdR could be directly converted to dUMP $\rightarrow$ dUDP $\rightarrow$ dUTP. Cells of tsA13 cdd-1 pyrG1 dck-4 were infected with PBS 1 at an MOI of 4 and labelled with either 6-^{3}H-uracil or 6-^{3}H-UdR. It was found that the fraction of the total CPM incorporated into DNA after the addition of 6-^{3}H-UdR was not significantly different from the fraction of CPM incorporated into DNA after the addition of 6-^{3}H-uracil (Table 29). This result indicates that no UdR kinase is induced in PBS 1-infected cells.

CdR kinase and CdR deaminase : Induction by PBS 1 of phage coded CdR kinase or CdR deaminase was tested by measuring

the incorporation of 5-^{3}H-CdR into nucleic acids of strain tsA13 cdd-1 pyrG1 dck-4. This strain carries the dck and cdd mutations and is unable to metabolise CdR either by CdR kinase or by CdR deaminase. If either CdR kinase or CdR deaminase is induced in phage infected mutant cells, 5-^{3}H-CdR must be incorporated into DNA or RNA. If only CdR kinase is induced, 5-^{3}H-CdR will be incorporated into DNA and if only CdR deaminase is induced, 5-^{3}H-CdR is expected to be incorporated mostly into RNA.

PBS 1-infected cells of tsA13 cdd-1 pyrG1 dck-4 were labelled with 5-^{3}H-CdR and it was found that no radioactivity was incorporated into nucleic acids (Table 30). This result indicates that neither CdR kinase nor CdR deaminase is induced after PBS 1 infection.

dCDP deaminase : In order to determine whether the host dCDP deaminase was affected by PBS 1 infection, the activity of this enzyme was assayed in cells of strain tsA13 cdd-1, from which many other strains used in this study were derived. The specific activity of dCDP deaminase was 70 nmoles/h/ mg protein in uninfected cells and 80 nmoles/h/ mg protein in PBS 1 infected cells of the same culture, indicating that dCDP deaminase was not affected by phage infection.

Ribonucleotide reductase : The activity of CTP reductase and UTP reductase were assayed in uninfected cells and in phage infected cells of strain dns-6 which has low levels of CTP reductase. It was found that both UTP reductase and CTP reductase activities remained practically the same during phage development (Table 31), indicating the absence of enzyme induction. It was also found that treatment with rifamycin which does not interfere with induction of phage coded enzymes (111, 115) caused a marked decrease in the specific activity of CTP reductase.

Activity of the thyB coded enzyme : It was found by Tomita and Takahashi (unpublished result) that PBS 1 infection does not alter the level of dTMP synthetase coded by the thyA locus. In order to investigate the thyB coded pathway in PBS 1-infected cells, the release of tritium from 5-^{3}H-dUMP was measured in crude extracts of uninfected and PBS 1-infected cells of strain tsA13 cdd-1 thyA thyB^{+}. In this strain the activity of the thyB coded pathway can be assayed without interference from the thyA coded thymidylate synthetase. Crude extracts were prepared from cells harvested before infection and 25 min after infection with PBS 1. These extracts were incubated with the standard reaction mixture. It was found that the amount of CPM released into the medium by extracts of infected cells was about 50 % of that released by uninfected cell extracts (Table 32).

Table 27.

Various types of cells in a PBS 1-infected culture.

Cell type	% of total in PA	% of total in MMC
phage producing	85	4
phage carrying	7	7
uninfected	7	7
"killed"	1	82

Cells of SB19E were grown in MMC and in PA to the late exponential phase and infected with PBS 1 at an MOI of 5. Ten min after infection the cultures were treated with phage antiserum (diluted 1:120) for 3 min. The number of surviving cells was determined on TBB. In order to establish the numbers of phage carrying and uninfected cells, individual colonies from the TBB plates were tested for their capacity to produce lysis on lawns of sensitive bacteria. The number of phage producing cells was calculated by subtracting the number of phage carrying cells from the total number of PFU determined 10 min after infection.

Table 28.

The effect of rifamycin on the rate of DNA synthesis in phage infected cells.

Phage	Relative rate of DNA synthesis	
	- rifamycin	+ rifamycin
PBS 1 (wild type)	1.00	0.77
SFU50	0.21	0.04
SNG5	0.25	0.07
No phage	0.92	0.07

Rifamycin (25 µg/ml) was added 2 min before infection. The rate of DNA synthesis was measured by the incorporation of 5-^{3}H-CdR. Radioactive material was added at 10 min after infection at a final concentration of 1 µCi/ml. The amount of radioactivity incorporated into TCA insoluble and alkali stable material was measured at 20 min after infection.

Table 29.

Incorporation of 6-^{3}H-UdR and 6-^{3}H-uracil into nucleic acids in infected cells.

	Time after the addition of UdR or uracil (min)	CPM in total nucleic acids	CPM in DNA	% of CPM in DNA
6-^{3}H-UdR	0	61	65	-
	7.5	5866	610	10.6
	15	12733	2137	16.8
6-^{3}H-uracil	0	169	191	-
	7.5	6206	568	9.2
	15	18973	4513	23.8

Cells of tsA13 cdd-1 pyrG1 dck-4 were grown in PA supplemented with 20 µg/ml CR. In the late exponential phase the cells were infected with PBS 1 at an MOI of 4. Twenty five min after infection 6-^{3}H-UdR or 6-^{3}H-uracil was added at a final concentration of 5 µCi/ml. The incorporation into nucleic acids, and DNA was measured at the times indicated.

Table 30.

Incorporation of 5-^{3}H-CdR into pyrimidine mutants infected with PBS 1.

	Time (min)	CPM incorporated in tsA13 cdd-1 pyrG1	CPM incorporated in tsA13 cdd-1 pyrG1 dck-4
Uninfected cells	0	35	13
	5	4286	25
	10	9366	46
	15	16011	49
	20	28258	29
PBS 1-infected cells	0	43	14
	5	7409	88
	10	13550	52
	15	22825	72
	20	30517	123

Cells of tsA13 cdd-1 pyrG1 and tsA13 cdd-1 pyrG1 dck-4 were grown in PA supplemented with 20 µg/ml CR. In the late exponential phase cells were infected with PBS 1 at an MOI of 4. Twenty min after infection 5-^{3}H-CdR was added at a final concentration of 1.25 µCi/ml. The incorporation of radioactivity into nucleic acids was measured in PBS 1-infected cells and in an uninfected control culture of both strains.

Table 31.

UTP reductase and CTP reductase activity before and after infection with PBS 1.

	UTP reductase specific activity[a]	CTP reductase specific activity[a]
Uninfected cells	6.6	436
PBS 1-infected cells	7.1	300
PBS 1-infected cell treated with rifamycin [b)]	4.0	54

[a] expressed as pmoles/h/ mg protein.

[b] 20 μg/ml rifamycin was added at 2 min before infection.

Table 32.

Activity of the thyB coded pathway after PBS 1 infection.

	Concentration of protein in the reaction mixture (mg/ml)	CPM in the supernatant liquid [a]	CPM in the control supernatant liquid [a]
Uninfected cells	2.5	11612	1975
	1.25	4760	893
PBS 1-infected cells	2.76	4499	1050
	1.38	2730	1129
Boiled extract	2.5	1530	615

5-^{3}H-dUMP was used as substrate in the assay, which was performed by the standard procedure.

[a] CPM in 0.5 ml of supernatant liquid. The CPM in the control are those released into the medium without incubation at 37 C for 3 min.

Figure 6.

Incorporation of ^{3}H-uridine into nucleic acids in PBS 1-infected cells.

A culture of SB19E (2×10^{8} cells/ml) in PA was infected with PBS 1 at an MOI of 4 at various times cells were pulse labelled with ^{3}H-UR (40 µCi/ml) for 5 min. The incorporation into nucleic acids and into DNA was measured.

Symbols:

○——○ , RNA.

○——○ , DNA.

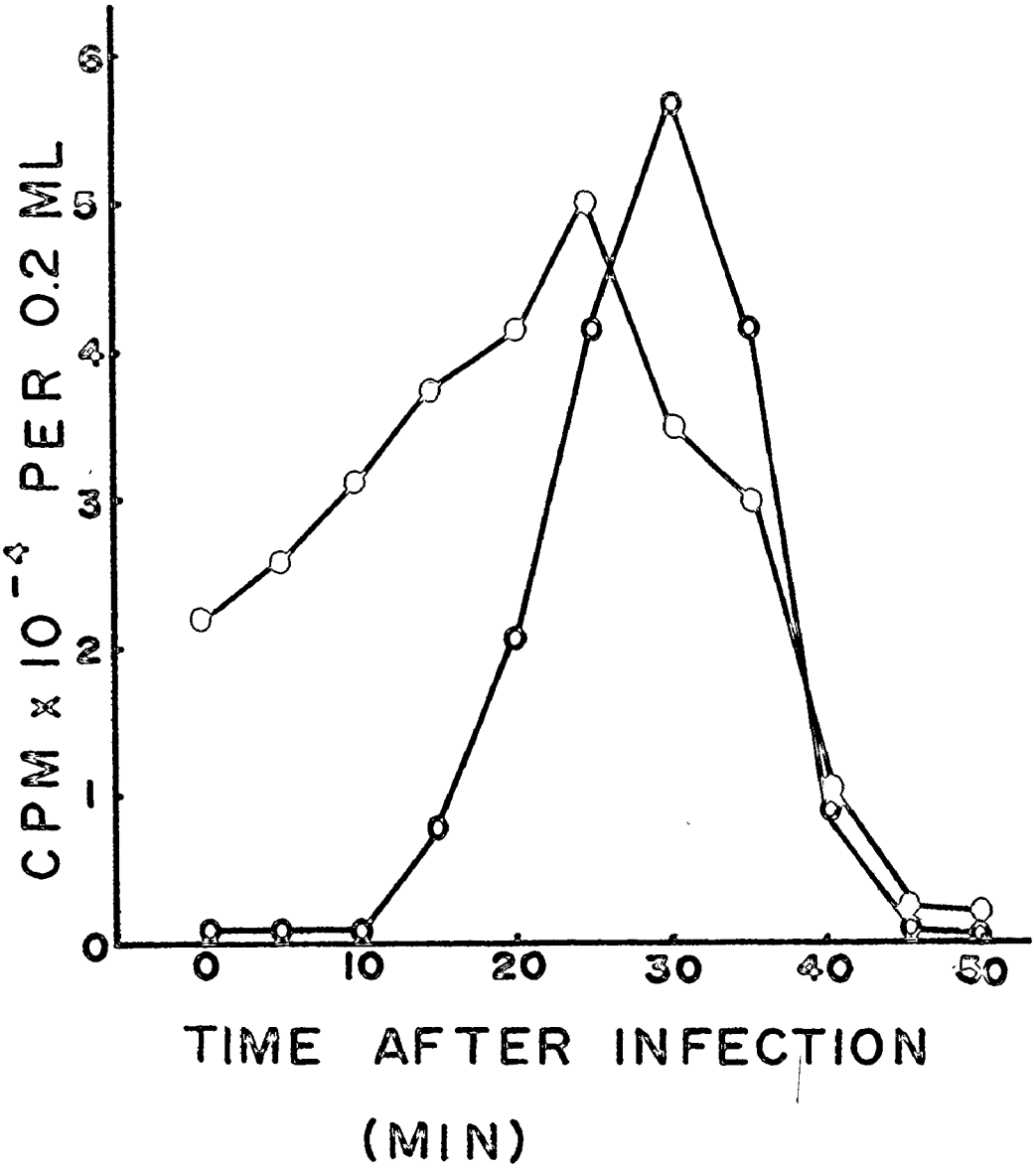

CPM x 10^{-4} PER 0.2 ML
0
1
2
3
4
5
6
0
10
20
30
40
50
TIME AFTER INFECTION
(MIN)

Figure 7.

Incorporation of ^{3}H-thymidine into nucleic acids in PBS 1-infected cells.

A culture of SB19E (2×10^{8} cells/ml) in PA was infected with PBS 1 at an MOI of 4 and labelled with methyl-^{3}H-TdR (100 µCi/ml) continuously. At various times after the addition of radioactive material the incorporation into nucleic acids was measured in the PBS 1-infected culture and in an uninfected control culture.

Symbols:

○——○ , uninfected cells.

○——○ , PBS 1-infected cells.

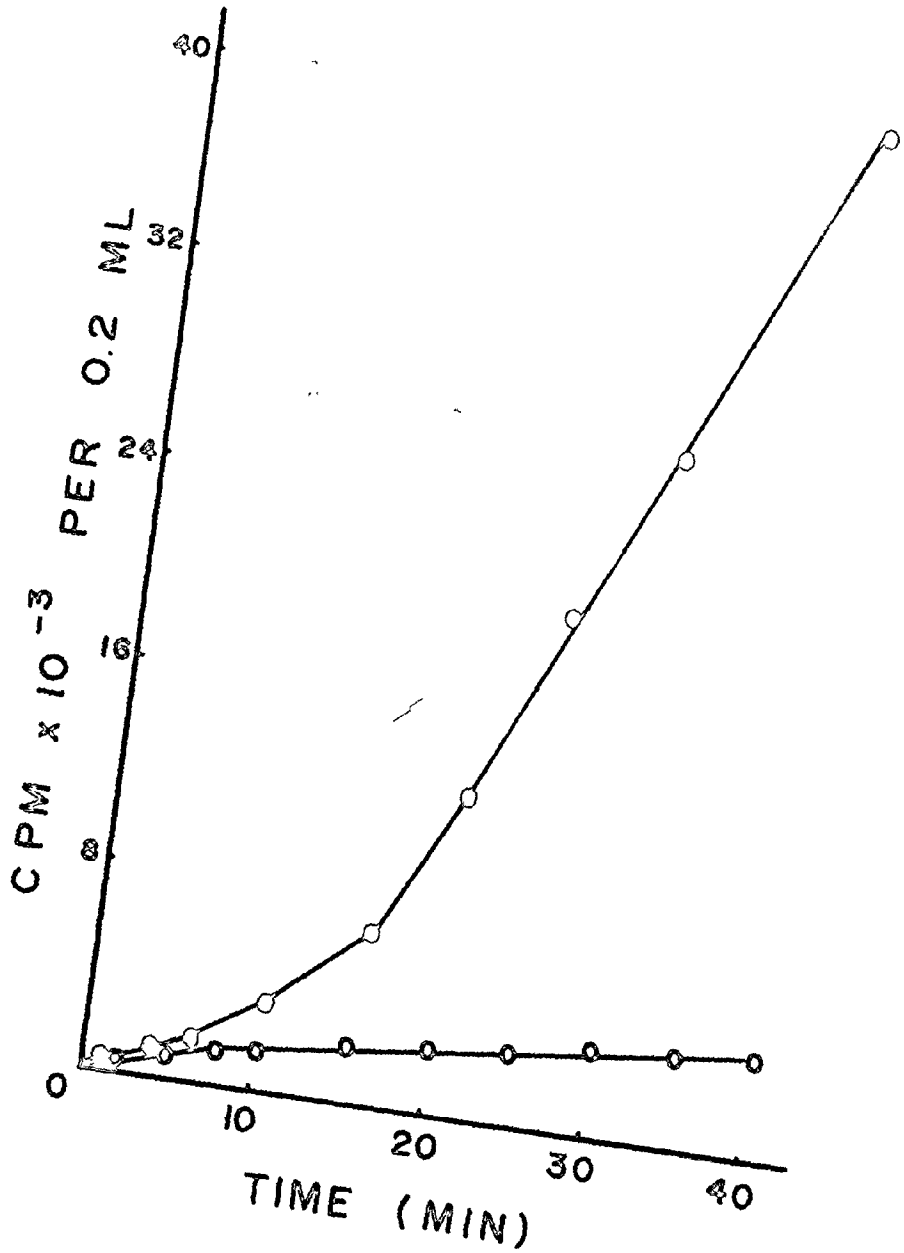

CPM x 10^-3 PER 0.2 ML
40
32
24
16
8
0
10
20
30
40
TIME (MIN)

Figure 8.

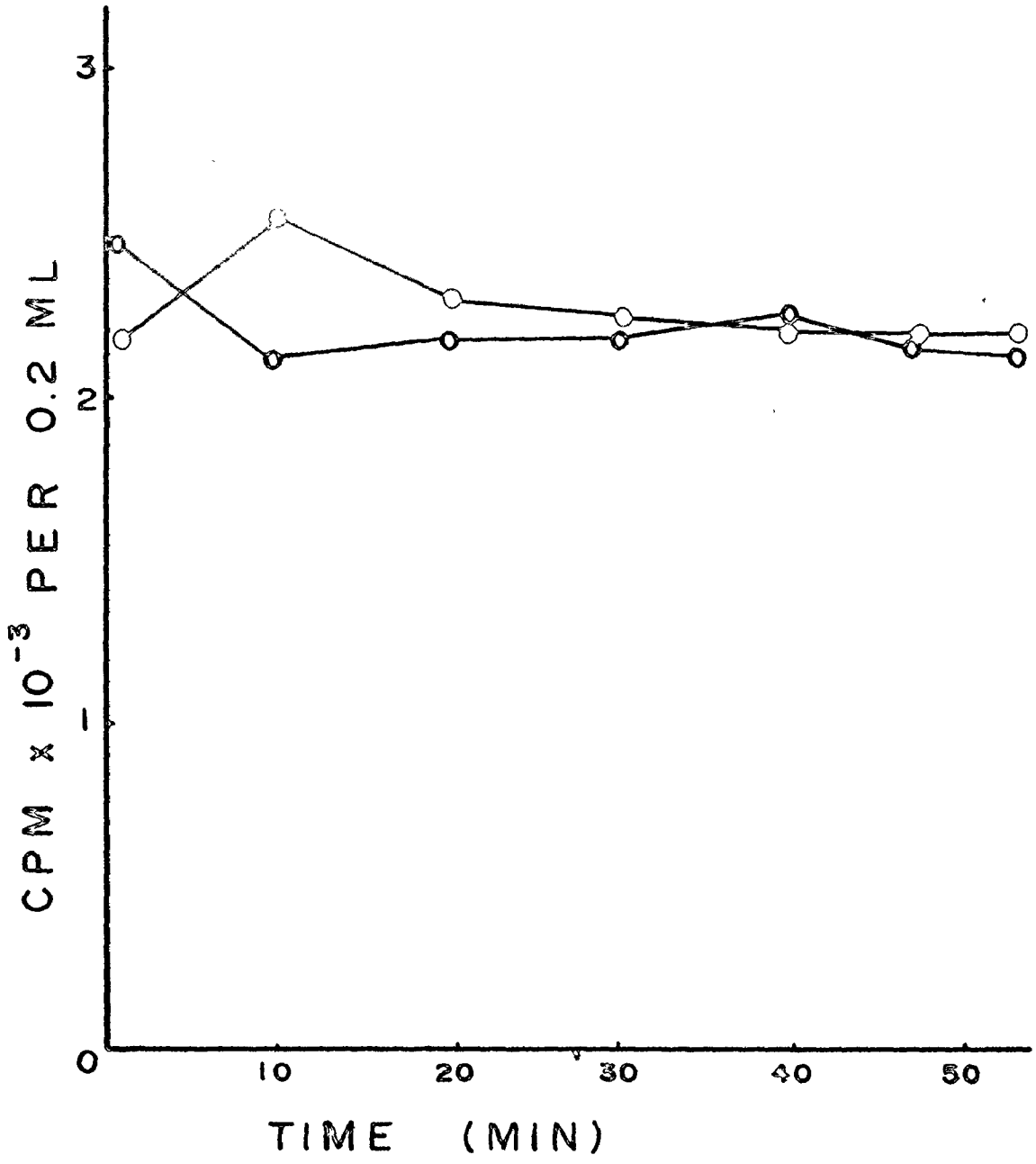
Stability of the host DNA in PBS 1-infected cells.

Cells of SB19E were prelabelled with methyl-^{3}H-TdR (40 µCi/ml) in PA in the late exponential phase for 1 hour. Cells were harvested by centrifugation at room temperature and diluted (1:10) in fresh prewarmed PA. One hour after resuspension of the cells the culture reached a turbidity of 80 Klett units and PBS 1 was added to an MOI of 5. At various times after infection the amount of TCA precipitable radioactivity was measured in a PBS 1-infected culture and in an uninfected control.

Symbols:

○——○ , uninfected cells.

o——o , PBS 1-infected cells.


CPM x 10^-3 PER 0.2 ML
3
2
1
0
10
20
30
40
50
TIME (MIN)

CHAPTER VI.

THE SYNTHESIS OF dUTP IN PBS 1-INFECTED CELLS.

The metabolism of pyrimidine compounds in B.subtilis and changes induced by PBS 1 infection investigated in this and earlier studies (59, 106, 137 - 139) are shown in Fig. 9. According to the proposed pathways dUTP, the precursor for PBS 1 DNA synthesis, can be formed either by reduction of UTP or by deamination of deoxycytidine nucleotides (dCTP and dCDP).

Fig. 10 shows the metabolism of pyrimidine compounds at later stages of PBS 1 development. It is assumed that dephosphorylation of dUDP and dUTP is reversed by phage induced dUMP kinase (59, 138) and that the synthesis of thymidine nucleotides is blocked as a consequence.

If one assumes that 1) the dCTP pool and the dUTP pool turnover rapidly and consequently the pools reach maximal specific activity rapidly after the addition of radioactive material, and that 2) the synthesis of deoxyribonucleotides in the PBS 1-infected cells is in a steady-state, the ratio $dUMP^{x}/dCMP^{x}$ in the phage DNA can be used to determine the fraction of dUTP derived from each pathway.

Although the validity of these assumptions has been verified for T4 infected cells of E.coli, no relevant data are available for B.subtilis.

In the present study the ratio $dUMP^x/dCMP^x$ was determined in the DNA of PBS 1 labelled with various bases and nucleosides in a number of mutant strains affected in the metabolism of pyrimidine.

On the basis of the above assumptions the ratio of the specific activity of dUTP to that of dCTP in the cell equals the ratio $dUMP^x/dCMP^x$ in the DNA. To interpret the results of the labelling experiments, the former ratio ($dUTP^x/dCTP^x$) was expressed as a function of the specific radioactivities of the substrates (UTP, dCTP and dCDP) and of the relative contribution of the pathways involved in the synthesis of dUTP (e.g. UTP reduction, dCTP deamination and dCDP deamination) as follows:

Let the fraction of dUTP derived from the reduction of UTP equal (x), and let the fraction derived from deamination of dCTP equal (y), then the fraction derived from the deamination of dCDP will be (1-x-y), assuming that the pathways in Figs. 9 and 10 are correct.

The specific activity of the dUTP pool (d) can be described as a weighted average of the specific activities of the UTP, dCTP and dCDP pools. Thus:

$$d = xa + yc + (1-x-y)e$$

where: d = the specific activity of the dUTP pool ,

a = the specific activity of the UTP pool ,

c = the specific activity of the dCTP pool ,

e = the specific activity of the dCDP pool ;

(x) = the fraction of dUTP derived from UTP reduction

(y) = the fraction of dUTP derived from dCTP deamination

(1-x-y) = the fraction of dUTP derived from dCDP deamination

The ratio d/c will be:

$$\frac{d}{c} = \frac{xa}{c} + y + \frac{(1-x-y)e}{c}$$

This ratio d/c equals the ratio $dUMP^{x}/dCMP^{x}$ in the DNA of PBS 1 which can be determined experimentally after labelling the DNA with various radioactive compounds.

This general equation can be simplified according to the mutants and radioactive compounds used:

When 5-^{3}H-CR or 5-^{3}H-CdR are used in strains carrying the cdd-1 mutation, no UTP will be labelled (Fig. 10). Therefore, the specific activity of UTP (a) will be zero and the term $\frac{xa}{c}$ can be omitted from the equation.

When 5-^{3}H-uracil or 5-^{3}H-UR are used in strains carrying the pyrG mutation, no cytidine or deoxycytidine nucleotides will be labelled (Fig. 10). Therefore, the specific activities of CTP (b), dCTP (c) and dCDP (e) will be zero and d/c will be infinite.

In strains lacking dCDP deaminase, the fraction (1-x-y) will be zero and x + y = 1.

In strains carrying the dck mutation, the specific activity of dCDP equals the specific activity of dCTP (e = c), since all dCDP is derived from dCTP in such a strain. Thus the general equation for such a strain becomes:

$$\frac{d}{c} = \frac{xa}{c} + (1-x)$$

In strains carrying a combination of the dck and crk mutations no dilution of the uridine and cytidine nucleotide and deoxynucleotide pools will occur (Fig. 10). The specific activities of UTP, CTP, dCTP, dCDP, dUDP and dUTP will be the same (a = b = c = d = e = f) and therefore d/c = 1.

No difference should be found in the value of d/c when DNA is labelled with 5-^{3}H-uracil or 5-^{3}H-UR, since both compounds enter the nucleotide pools via UMP.

A summary of the modifications of the general formula expressing the ratio d/c is shown in Table 33.

Various mutants infected by PBS 1 were labelled with 5-^{3}H-uracil, 5-^{3}H-CR, 5-^{3}H-CdR or in one case 5-^{3}H-UdR and the ratio dUMP^x/dCMP^x was determined (Table 34).

The ratio dUMP^x/dCMP^x was found to be 1.0 in strain tsA13 cdd-1 dck-3 crk-1 when this strain was labelled with either 5-^{3}H-uracil or 5- H-UR after infection.

The dCMP in phage DNA was not labelled when cells of tsA13 cdd-1 pyrG1 dck-4 were infected and labelled with 5-^{3}H-uracil. The ratio dUMP^x/dCMP^x in the phage DNA was very high.

If UdR kinase contributes to the dUTP pool, a high ratio dUMP^x/dCMP^x would be expected, since deoxyuridine nucleotides would be labelled directly while deoxycytidine nucleotides would remain unlabelled. However, it was found that the ratio dUMP^x/dCMP^x in PBS 1 DNA labelled with 5-^{3}H-UdR in wild type cells was the same as the ratio obtained with 5-^{3}H-UR. This result indicates that UdR is broken down to uracil and dR-1-P and that the radioactivity enters the nucleotide pools via UMP. This result is thus in agreement with earlier data shown in Tables 2, 4, 5 and 26 which indicate that UdR is predominantly metabolized by conversion to uracil and dR-1-P.

If a large amount of 5-^{3}H-CdR is metabolized via UdR by deamination, the ratio dUMP^x/dCMP^x in DNA labelled with 5-^{3}H-CdR in wild type cells will be similar to those obtained with 5-^{3}H-UdR and 5-^{3}H-UR. However, the ratio dUMP^x/dCMP^x in the DNA of PBS 1 labelled with 5-^{3}H-CdR was different from the ratios obtained with 5-^{3}H-UdR or 5-^{3}H-UR (Table 34). Furthermore, the ratio dUMP^x/dCMP^x in phage DNA labelled in wild type cells would be expected to be different from the ratio obtained in a cdd mutant if a large amount of CdR were deaminated in wild type cells.

However, no difference was found between the ratio obtained with 5-^{3}H-CdR in cdd mutants and in wild type cells (Table 34). These results indicate that CdR is predominantly metabolized by conversion to dCMP in wild type cells and not by deamination. This is in agreement with incorporation experiments carried out earlier which showed that more than 95 % of the 5-^{3}H-CdR incorporated into nucleic acids in wild type cells was incorporated into DNA rather than into RNA.

The ratio $dUMP^x/dCMP^x$ in PBS 1 DNA labelled with 5-^{3}H-CR in strain tsA13 cdd-1 dck-3 was 0.53. This indicated that in this strain the value of (1-x) was 0.53 (Tables 33 and 34) and consequently (x) was 0.47. Therefore, about 50 % of the dUTP was derived from reduction of UTP and the remainder was derived from deamination of dCDP and dCTP.

The values of the ratio $dUMP^x/dCMP^x$ in strain tsA13 cdd-1 ddd-3 labelled with 5-^{3}H-CdR or 5-^{3}H-CR were 0.44 and 0.58 respectively. The value of the ratio $dUMP^x/dCMP^x$ in tsA13 cdd-1 ddd-3 crk-3 labelled with 5-^{3}H-CdR was 0.47. Tables 33 and 34 show that these values are indicative of the value of (y), the fraction of dUTP derived from deamination of dCTP, in these strains. The average value of (y) in the three determinations was 0.50. Since in the ddd mutants (1-x-y) equals zero, (x+y) equals 1. It may be concluded that in these strains about 50 % of dUTP is derived from reduction of UTP and about 50 % is derived from deamination of dCTP.

It was observed that the value of (x) was about 0.50 in strains tsA13 cdd-1 dck-3, tsA13 cdd-1 ddd-3 and tsA13 cdd-1 ddd-3 crk-3. If we assume that (x) is also about 0.50 in strain tsA13 cdd-1, the values of (y) and (1-x-y) can be estimated in this strain.

The general formula for the ratio $dUMP^{*}/dCMP^{*}$ in PBS 1 DNA labelled in strain tsA13 cdd-1 with 5-^{3}H-CdR or with 5-^{3}H-CR is:

$$\frac{d}{c} = y + \frac{(1-x-y)e}{c}$$

Since (x), (y) and (1-x-y) are constant for a given strain, the observation that the values of d/c of PBS 1 DNA in strain tsA13 cdd-1 labelled with 5-^{3}H-CdR was different from that obtained with 5-^{3}H-CR was unexpected. The parameter which causes this difference is the value of e/c, which is the ratio of the specific activity of dCDP to that of dCTP. In experiments on the synthesis of dTTP in uninfected cells, it was already observed that the ratio $dCDP^{*}/dCTP^{*}$ (e/c) depended on the type of label used (Table 26). A similar difference between the values obtained with ^{14}C-CdR and ^{14}C-CR was observed.

The above equation can be rewritten to:

$$\frac{e}{c} = \frac{\frac{d}{c} - y}{1-x-y}$$

The values of d/c in PBS 1 DNA labelled with 5-^{3}H-CdR and 5-^{3}H-CR were found to be 0.97 and 0.50, respectively (Table 34). Thus, if we introduce these experimental values

for d/c in the formula we obtain:

$$\left(\frac{e}{c}\right)_{CR} = \frac{0.50 - y}{1-x-y} \quad \text{and} \quad \left(\frac{e}{c}\right)_{CdR} = \frac{0.97 - y}{1-x-y}$$

and consequently, since (x) , (y) and (1-x-y) are constant in one strain:

$$\frac{\left(\frac{e}{c}\right)_{CdR}}{\left(\frac{e}{c}\right)_{CR}} = \frac{0.97 - y}{0.50 - y}$$

The parameter $\left(\frac{e}{c}\right)_{CdR} / \left(\frac{e}{c}\right)_{CR}$ is determined by the degree at which the specific activities of the dCTP and the dCDP pool are diluted by unlabelled compounds derived from the CTP reductase or CdR kinase pathways (CdR to dCDP). If we assume that the degree at which the dCTP and dCDP pools are diluted by unlabelled material remains constant after infection, then we can substitute the value of $\left(\frac{e}{c}\right)_{CdR} / \left(\frac{e}{c}\right)_{CR}$ by the value of this parameter in uninfected cells which can be calculated from the ratios $dTMP^x/dCMP^x$ in DNA of strain <u>ts</u>A13 <u>cdd</u>-1 labelled with ^{14}C-CdR and ^{14}C-CR (Table 26).

The value of $\left(\frac{e}{c}\right)_{CdR} / \left(\frac{e}{c}\right)_{CR}$ would equal:

$$\frac{(dTMP^x/dCMP^x)_{CdR}}{(dTMP^x/dCMP^x)_{CR}} = \frac{0.62}{0.19} \approx 3$$

Therefore: $\frac{0.97 - y}{0.50 - y} = 3$ and $y = 0.26$.

If $x = 0.50$, $1-x-y = 0.24$.

Therefore in strain <u>ts</u>A13 <u>cdd</u>-1 , about 50 % of dUTP may be derived from the reduction of UTP, 25 % may be derived from deamination of dCTP and 25 % may be derived from deamination of dCDP.

Table 33.

Modification of the general formula describing the ratio of $dUMP^{x}$ to $dCMP^{x}$.

Strain	Radioactive compound added		
	5-^{3}H-U or 5-^{3}H-UR	5-^{3}H-CR	5-^{3}H-CdR
tsA13 cdd-1 dck-3 crk-1	$\frac{d}{c} = 1.00$	impossible	impossible
tsA13 cdd-1 pyrG1 dck-4	$\frac{d}{c} =$ infinite	$\frac{d}{c} = y + \frac{(1-x-y)e}{c}$	impossible
SB19E (wild type)	$\frac{d}{c} = \frac{xa}{c} + y + \frac{(1-x-y)e}{c}$	$\frac{d}{c} = \frac{xa}{c} + y + \frac{(1-x-y)e}{c}$	$\frac{d}{c} = \frac{xa}{c} + y + \frac{(1-x-y)e}{c}$
tsA13 cdd-1	$\frac{d}{c} = \frac{xa}{c} + y + \frac{(1-x-y)e}{c}$	$\frac{d}{c} = y + \frac{(1-x-y)e}{c}$	$\frac{d}{c} = y + \frac{(1-x-y)e}{c}$
tsA13 cdd-1 dck-3	$\frac{d}{c} = \frac{xa}{c} + (1-x)$	$\frac{d}{c} = 1-x$	impossible
tsA13 cdd-1 ddd-3	$\frac{d}{c} = \frac{xa}{c} + y$	$\frac{d}{c} = y$	$\frac{d}{c} = y$
tsA13 cdd-1 ddd-3 crk-3	$\frac{d}{c} = \frac{xa}{c} + y$	impossible	$\frac{d}{c} = y$

Table 34.

The ratios of dUMP* to dCMP* in PBS 1 DNA.

Strain	Radioactive compound added [a]			
	5-^{3}H-uracil	5-^{3}H-CR	5-^{3}H-CdR	5-^{3}H-UR
tsA13 cdd-1 dck-3 crk-1	0.98 ± 0.04	impossible	impossible	0.96 ± 0.10 ○
tsA13 cdd-1 pyrG1 dck-4 [c]	150 [d,○]	not determined	impossible	not determined
SB19E (wild type)	1.59 [b,d,○]	not determined	0.94 [d]	1.61 [d]
tsA13 cdd-1	1.59 ± 0.09	0.50 ± 0.02	0.97 ± 0.04	not determined
tsA13 cdd-1 dck-3	1.45 ± 0.12 ○	0.53 ± 0.05	impossible	not determined
tsA13 cdd-1 ddd-3	1.21 ± 0.06	0.44 ± 0.02	0.58 ± 0.06	not determined
tsA13 cdd-1 ddd-3 crk-3	0.89 ± 0.10 ○	impossible	0.47 ± 0.04	not determined

Legend: see page 129

The ratio $dUMP^{x}/dCMP^{x}$ was calculated from the ratio of CPM in dUMP/dCMP. The total CPM on the chromatograms ranged from 4000 - 50,000 , except for those ratios indicated with ⊗ in which 1500 - 2500 CPM were used.

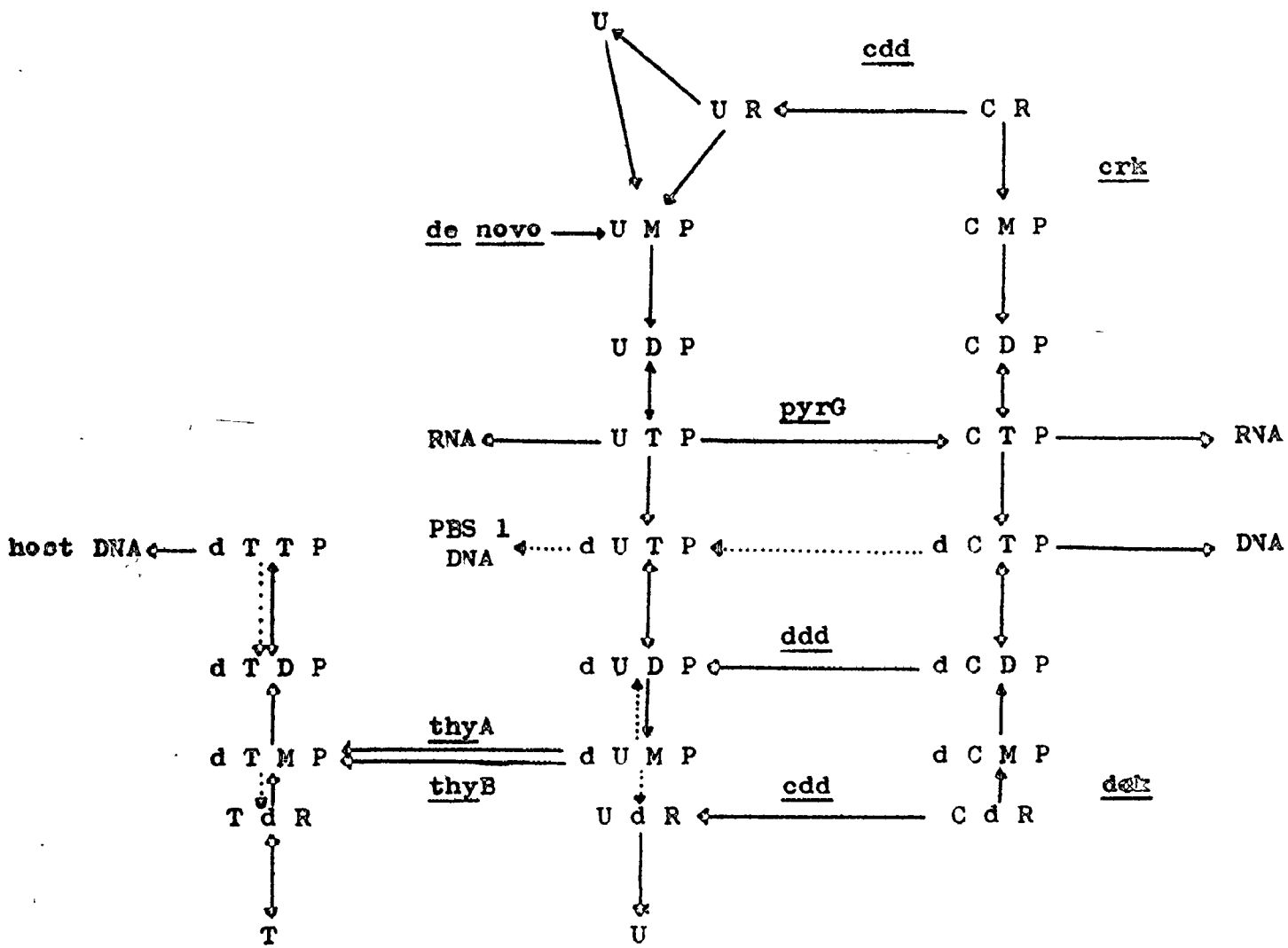
[a] 5-^{3}H-uracil and 5-^{3}H-CR were added at a final concentration of 5 µCi/ml; 5-^{3}H-CdR was added at a concentration of 1 µCi/ml and 5-^{3}H-UR was added at a concentration of 10 µCi/ml.

[b] 5-^{3}H-UdR (10 µCi/ml) was used in this experiment.

[c] All experiments were carried out in PA,except for this strain 20 µg/ml CR was added to the medium.

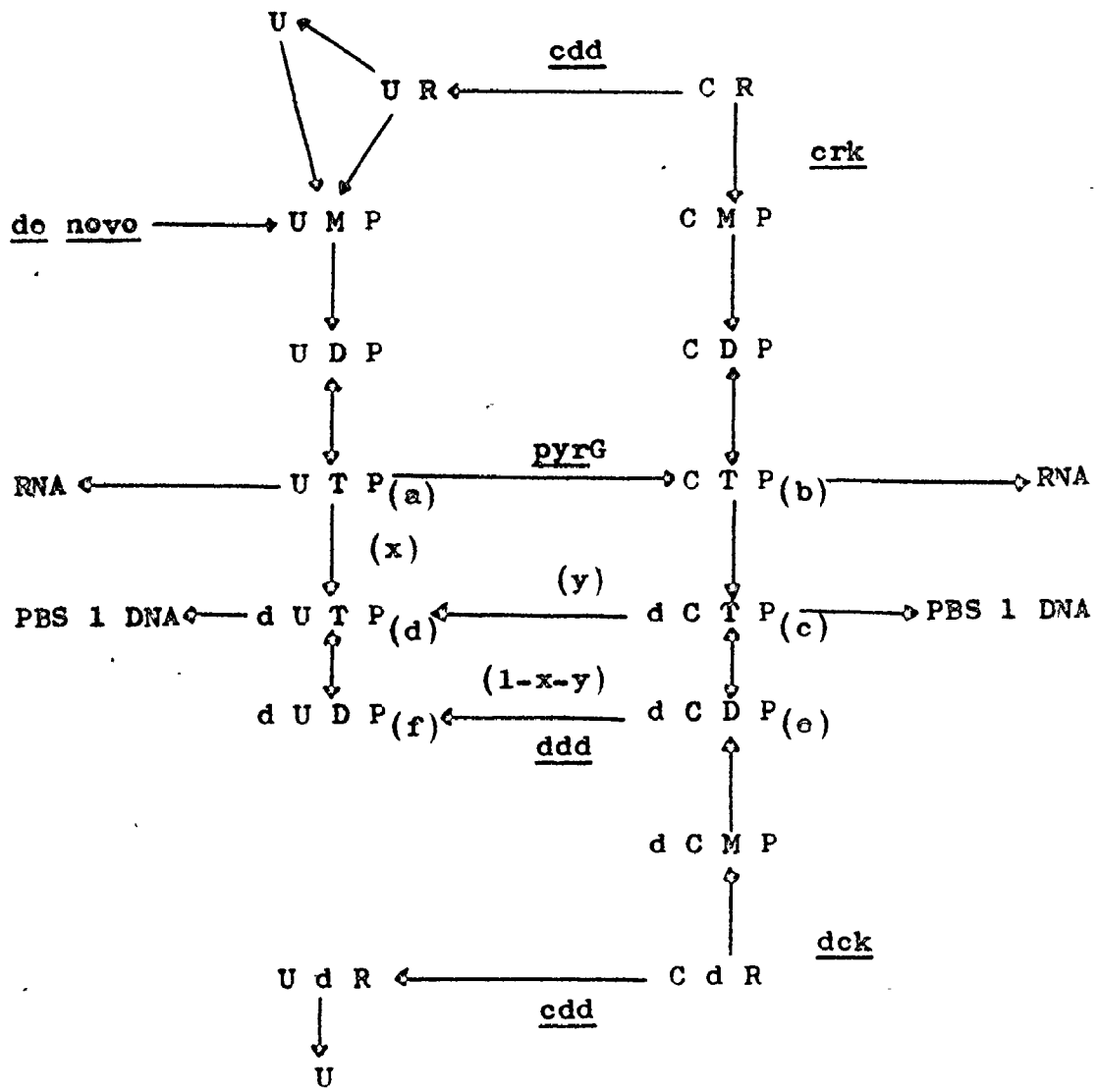
[d] These values are averages of two determinations.

Figure 9 : PYRIMIDINE METABOLISM IN BACILLUS SUBTILIS



PBS 1 induced enzyme activity: ·····→

Figure 10 : METABOLISM OF PYRIMIDINE NUCLEOTIDES ALTERED BY PBS 1 INFECTION



CHAPTER VII.

DISCUSSION.

Metabolism of pyrimidine bases and nucleosides in uninfected cells.

The foregoing results indicate that the metabolism of uracil, UR and UdR in B.subtilis resembles that in enteric bacteria. Uracil and UR are converted to UMP and serve as a precursor for the pyrimidine compounds required by the cell (6, 19).

The observation that the incorporation of radioactive UR into nucleic acids was significantly lower than that of uracil, suggests that the conversion of uracil to UMP does not involve UR as an intermediate. An alternative explanation may be that the poor incorporation of UR is due to poor uptake of this compound by the cells. In E.coli and S.cerevisiae, however, the uptake does not seem to be the rate limiting step in the incorporation of bases and nucleosides into nucleic acids (47, 62, 94, 103). In B.subtilis, therefore, uracil may also be converted to UMP by an enzyme comparable to the E.coli UMP pyrophosphorylase which links uracil to phosphoribosyl pyrophosphate (21, 97).

It is not known whether UR is metabolized in B.subtilis by both UR kinase and UR phosphorylase as in the case of E.coli and S.typhimurium (9, 97).

The metabolism of UdR in B.subtilis seems to be similar to that in enteric bacteria. UdR is converted to uracil and deoxyribose-1-phosphate in the cell and the activity of UdR kinase which can be demonstrated in vitro does not seem to play an important role in the metabolism of UdR. Beck et al. (9) and Fuchs and Neuhard (40) report that the conversion of UdR to dUMP is not significant in enteric bacteria unless the bacterial strains used are deficient in TdR phosphorylase (tpp). This enzyme breaks down TdR and UdR to the corresponding bases and dR-1-P. Beck et al. (9) found that in tpp^+ strains of S.typhimurium FUdR was broken down so rapidly that no toxic intracellular level of FUdR can be maintained.

The metabolism of cytosine, CR and CdR in B.subtilis is similar in some aspects and different in others to that in enteric bacteria. Both B.subtilis and the enteric bacteria contain deoxycytidine-cytidine deaminase, which allows pyrimidine requiring mutants to grow on CR or CdR as pyrimidine source (9, 97). While in enteric bacteria CdR is the preferred substrate for this deaminase (97), in B.subtilis CdR and CR appear to be equally good substrates. The activity of CdR deaminase found in this

study was similar to that obtained by Tomita and Takahashi (137, 138).

Unlike the enteric bacteria, B.subtilis contains an active CdR kinase, which phosphorylates CdR. The presence of this enzyme was suggested by Bazill and Karamata (8) from their study of mutants affected in ribonucleotide reduction. In the present study, the presence of the enzyme was demonstrated by conversion of CdR to dCMP in vitro. CdR appears to be metabolized predominantly by conversion to dCMP by CdR kinase, rather than by demination. This is suggested by the observation that more than 95 % of the radioactivity from 5-^{3}H-CdR incorporated into nucleic acids was present in DNA.

A high level of CR kinase activity was found in B.subtilis. No such enzyme has been described for other organisms. In enteric bacteria CR is converted to CMP by UR kinase (9, 97). CR kinase in B.subtilis is distinct from CdR kinase, since dck mutants which lack CdR kinase are not affected in their capacity to incorporate 5-^{3}H-CR into nucleic acids. The enzyme is also distinct from UR kinase, since crk mutants which are blocked in the incorporation of radioactive CR are still able to incorporate radioactive UR into nucleic acids at a rate comparable to that of wild type cells. An alternative explanation may be that the incorporation of radioactive UR in crk mutants is mediated by UR phosphorylase. It is thus not completely

excluded that UR kinase and CR kinase are the same enzyme. To determine whether UR kinase and CR kinase are distinct enzymes, uridine phosphorylase mutants (upp) will have to be isolated and sensitivity of mutants of the type crk upp to FUR must be tested.

In contrast to the pyrimidine requiring mutants of enteric bacteria, mutants in B.subtilis cannot use cytosine as sole source of pyrimidine. This indicates that cytosine cannot be deaminated by B.subtilis in sufficient amounts to allow growth of pyr mutants. Like in E.coli and S.typhimurium (9, 91, 97), in B.subtilis strains carrying the pyrG mutation CR cannot be replaced by the addition of cytosine to the medium. This indicates that neither CR phosphorylase nor a CMP pyrophosphorylase are present in this organism.

Trans-N-deoxyribosylase which is present in Lactobacilli (11) seems to be absent in B.subtilis. Some tsA mutants of Bazill and Karamata (8) required two deoxyribonucleosides for growth and, one deoxyribonucleoside and the three other bases did not support growth of those mutants. The dns mutants isolated in this study showed similar requirements for CdR and one of the purine deoxyribonucleosides. If trans-N-deoxyribosylase is active in B.subtilis one deoxyribonucleoside plus the other bases should be sufficient to supply the cells with all deoxyribonucleosides.

Metabolism of pyrimidine nucleotides in uninfected cells.

It is clear from this study that strains carrying the pyrG mutation are blocked in the conversion of uridine ribonucleotides to cytidine ribonucleotides. A number of pyrG mutants reverted to CR independence. This may indicate that only one reaction is involved in the amination of uridine nucleotides. It is unlikely that the pyrG mutants originally are double mutants and that single revertants are CR independent analogous to the case of thymine dependence in B.subtilis. The pyrG mutants are most likely blocked in the conversion of UTP to CTP by CTP synthetase. Although no biochemical evidence is presented, it is assumed that the amination of uridine ribonucleotides takes place at the triphosphate level in B.subtilis. In all other organisms studied so far the amination of uridine ribonucleotides takes place at the triphosphate level (97).

The observations of Wilson et al. (150) on genetic loci which govern the biosynthesis of thymidine nucleotides in B.subtilis were confirmed in this study with strain 167 tsA13 thyA thyB and its cdd-1 derivative. Aminopterin resistant and sensitive thy$^+$ transformants were isolated and sensitivity to aminopterin was correlated with the presence of the thyA coded thymidylate synthetase in the various strains. The observation that dTMP in B.subtilis DNA is labelled by radioactive CdR suggests the existence of a pathway for the conversion of deoxycytidine nucleotides

to dTTP. The values obtained for the ratio $dTMP^{x}/dCMP^{x}$ in the DNA of mutants with various combinations of the thyA and thyB genotypes indicate that a deoxyuridine nucleotide is an intermediate in this conversion of deoxycytidine nucleotides to dTTP. An enzyme activity which released tritium from the 5-position of 5-^{3}H-dUDP was present in extracts of strain thyA $thyB^{+}$ and absent in strain thyA thyB. Since it was found that tritium was released in equal amounts from 5-^{3}H-dUMP and 5-^{3}H-dUDP and since the latter compound was rapidly and quantitatively converted to dUMP, it was concluded that dUMP was the substrate for the enzyme coded by the thyB locus.

The foregoing conclusion that the conversion of a deoxycytidine nucleotide to dTTP occurs with a deoxyuridine nucleotide as intermediate implies that B.subtilis cells must contain a deoxycytidine nucleotide deaminase. Indeed, a deaminase for dCDP was found in B.subtilis cells and a preliminary characterisation of this enzyme was carried out.

A number of mutants lacking dCDP deaminase (ddd) were isolated in the course of this study. They were FCdR resistant derivatives of strains already carrying the cdd-1 mutation. The ddd mutants differ from dck mutants, which are also resistant to FCdR, in the rate of incorporation of radioactive CdR into nucleic acids. The ddd mutants incorporate radioactive CdR at reduced rates, possibly due to a reduced rate of turnover of the dCDP pool.

Results of experiments on ribonucleotide reductase in crude extracts of B.subtilis were variable and inconclusive. Similar difficulties were encountered in the early work on ribonucleotide reductase in crude extracts of E.coli and L.leichmannii (73). Larsson and Reichard (73) in a review of the earlier experiments on this enzyme in crude extracts of E.coli state that "the activities of different extracts varied wildly, and the results of kinetic experiments were very peculiar". Contrasting evidence on the role of ATP and Mg^{++} accumulated in studies on ribonucleotide reductase in crude extracts of L.leichmannii (33, 73). Beck (10) showed in a study on the regulation of this enzyme that a very complex interplay exists between ATP and divalent cations, which can be stimulatory or inhibitory depending on the substrate and the concentration and type of effector molecules present (ATP or deoxyribonucleoside triphosphates).

It is assumed in the present and other studies (8) that the reduction of ribonucleotides in B.subtilis is carried out by one enzyme. No real evidence for this assumption exists, except that in all other organisms studied so far only one enzyme is responsible for the reduction of ribonucleotides. The existence of only one ribonucleotide reductase in B.subtilis is suggested by the study of Bazill and Karamata (8) on tsA mutants and by our study on dns mutants. Both types of mutants require a pyrimidine and a purine deoxyribonucleoside for growth. It seems therefore

that these mutants are affected in one enzyme that reduces both purine and pyrimidine ribonucleotides.

Although the ribonucleotide reductase activity observed was variable, the rate of reduction of cytidine ribonucleotides was consistently greater than that of uridine ribonucleotides. Similar observations were made in crude extracts of E.coli and L.leichmannii. In these organisms the rate of reduction in vitro of uridine ribonucleotides was markedly lower than that of reduction of cytidine ribonucleotides (10, 16, 43). Results presented in this thesis support the hypothesis that ribonucleoside triphosphates are the substrates for ribonucleotide reductase in B.subtilis. If we assume that the reductase acts on either CDP or CTP but not on both, the following observation would also suggest that the reduction takes place at the triphosphate level. If B.subtilis had a diphosphate reductase, then both radioactive CdR and radioactive CR would label dCDP before dCTP. From the dCDP pool the radioactivity would flow to dCTP and dTTP in uninfected cells and to dCTP and dUTP in PBS 1-infected cells (Figs. 5 and 9). One would not expect any difference in the ratio $dTMP^{x}/dCMP^{x}$ in uninfected cells of cdd mutants or in the ratio of $dUMP^{x}/dCMP^{x}$ in PBS 1-infected cells of cdd mutants between DNA labelled with radioactive CdR and DNA labelled with radioactive CR. However, such differences were found in the present study (Tables 26 and 34). The above statement is valid only if deoxyribonucleoside triphosphates are the immediate precursors

for DNA, and if no part of the dCDP pool is channelled specifically to either dCTP or dTTP (dUTP), analogous to the channelling of carbamyl phosphate to arginine or to pyrimidine biosynthetic pathways in N.crassa (28).

B.subtilis cells are sensitive to hydroxyurea (99). Growth inhibition of B.subtilis can be overcome by deoxyribonucleosides only in strains which can convert these compounds to deoxyribonucleotides. Therefore, the deoxyribonucleosides seem to exert their effect in overcoming the inhibition by hydroxyurea after conversion to deoxyribonucleotides. Thus, our results on growth inhibition suggested that the ribonucleoside triphosphate reductase in B.subtilis might be sensitive to hydroxyurea, as in the case of E.coli (122). However, ribonucleoside triphosphate reductase activities were not inhibited by this drug. Ribonucleoside triphosphate reductase of B.subtilis, therefore, resembled the L.leichmannii enzyme (33).

Our dns mutants which require deoxyribonucleosides for growth and the tsA13 mutant of Bazill and Karamata (8) were not deficient in CTP reductase and UTP reductase activity. Some of the dns mutants were temperature-sensitive in their requirement for deoxyribonucleosides and tsA13 mutation could be suppressed by the introduction of other mutations (cdd-1 thyB or cdd-1 ddd-3). These observations indicate that the requirement for deoxyribonucleosides may be associated with relative growth rate as in the nrd mutant

of E.coli (41). It may also be due to altered regulatory properties of ribonucleotide reductase in these mutants, rather than defectiveness in ribonucleotide reductase.

The synthesis of dTTP.

The synthesis of dTTP in B.subtilis was studied by determining the ratio $dTMP^{x}/dCMP^{x}$ in the DNA of various mutants labelled with radioactive uracil, CR or CdR for more than two generations. This ratio should be a measure of the amount of dTTP derived from the various pathways involved in the synthesis of this compound. However, the validity of relating the ratio $dTMP^{x}/dCMP^{x}$ in the DNA to the ratio of the specific activities in the pools of DNA precursors rests on the following assumptions.

The first assumption is that steady-state conditions exist with regard to the synthesis of deoxyribonucleotides in B.subtilis. It was shown by Neuhard and Thomassen (92) that steady-state conditions exist in exponentially growing cultures of E.coli, since the pool sizes of the various deoxyribonucleoside triphosphates remain constant throughout growth. Similar studies with B.subtilis have not yet been made.

The second assumption is that the pools of dCTP and dTTP turnover rapidly, so that they reach maximum specific activity soon after the addition of radioactive compounds. According to Neuhard and Thomassen (92) the

dCTP pool in E.coli had a half time of 1.1 min at 30 C. The turnover rate at 37 C was too fast to be measured accurately. According to Werner (146) maximum specific activity of the dTTP pool was reached within 5 min in E.coli growing with a generation time of 400 min; i.e., within 1/80 th. of the generation time maximum specific activity was reached. It seems thus valid to assume that the pools of deoxyribonucleotides reach maximum specific activity rapidly enough that the initial imbalances will not affect the overall ratio of the specific activities established over a period of more than two generations of the cells.

The third assumption is that there is no compartmentalisation or channelling in the pools of deoxyribonucleotides. It is assumed that all deoxyribonucleotides participate with equal probability in the DNA polymerisation process or in the formation of other deoxyribonucleotides. Evidence suggestive of compartmentalisation in DNA synthesizing systems in freeze-treated B.subtilis was presented by Billen et al. (17, 18). However, this evidence was inconclusive since no differentiation could be made between incorporation into viable and non-viable cells in the population of freeze-treated cells.

The fourth assumption is that deoxyribonucleoside triphosphates are the immediate precursors for DNA synthesis. This was questioned by Werner (146) in 1971. A

postulated precursor dTMP-X, synthesized from dTDP was proposed as immediate precursor for DNA replication, while dTTP was suggested to be the precursor for repair synthesis. The assumption that deoxyribonucleoside triphosphates are immediate precursors for DNA synthesis can not be proven at this time. However, the results in vitro with DNA polymerase III in E.coli and in B.subtilis suggest that this enzyme, which is indicated to be the replicative enzyme, requires deoxyribonucleoside triphosphates for activity (44, 148, 149). The following observations made in this present study indicate that deoxyribonucleoside triphosphates may be the precursors for DNA synthesis, although deoxyribonucleoside monophosphates as immediate DNA precursors are not ruled out entirely. It was found that the ratio $dTMP^{x}/dCMP^{x}$ in DNA of cdd mutants labelled with radioactive CR was different from that labelled with radioactive CdR. If the diphosphates are the precursors for DNA synthesis, these ratio are not expected to be different.

If we accept these assumptions, the ratio $dTMP^{x}/dCMP^{x}$ is indicative of the amount of dTTP derived from the various pathways. In MMC about 40 % of the dTTP was found to be derived from deamination of deoxycytidine nucleotides. This conclusion was based on the observation that the ratio $dTMP^{x}/dCMP^{x}$ in DNA labelled with ^{14}C-CR was 0.39 in strain tsA13 cdd-4 grown in MMC. The remainder of the dTTP must

have been synthesized from reduction of UTP, since in MMC no exogenous TdR or CdR are present.

In PA the contribution of various pathways could not be established rigorously. The results in Table 26 suggested that in strain tsA13 cdd-1 dck-3 crk-1 about 17 % of the dTTP was derived from exogenous TdR. In strain tsA13 cdd-1 20 % of the dTTP was derived from reduction of CTP. If we assume that the overall contribution of the dCDP deaminase pathway to the synthesis of dTTP was the same as in the case of MMC and that about 17 % of the dTTP was derived from exogenous TdR in strain tsA13 cdd-1, we can draw the following conclusion: 17 % of dTTP was derived from exogenous TdR in strain tsA13 cdd-1, 20 % was derived from reduction of CTP, 20 % was derived from exogenous CdR and the remainder (43 %) was derived from reduction of UTP.

Pyrimidine metabolism in PBS 1-infected cells.

In PBS 1-infected cells no appreciable incorporation of 6-^{3}H-UdR into DNA through dUMP was detectable. Thus, it appears that UdR does not contribute directly to the intracellular pools of deoxyuridine nucleotides in PBS 1-infected cells.

Radioactive CdR could not be metabolised by PBS 1-infected mutants which lack CdR kinase and CdR deaminase. This indicates that PBS 1 does not induce CdR kinase or CdR deaminase.

The specific activity of dCDP deaminase showed no change after PBS 1 infection, so this enzyme may be involved in the synthesis of deoxyuridine nucleotides in the phage infected cells.

In T4-infected cells of E.coli, a phage coded ribonucleoside diphosphate reductase was found (157). In PBS 1-infected cells the specific activity of UTP reductase and CTP reductase remained at the same level. This might reflect the absence of PBS 1 coded ribonucleotide reductase. An alternative explanation for this observation would be that our assay method could not detect the phage specific ribonucleotide reductase.

Tomita and Takahashi (unpublished result) found that the specific activity of thymidylate synthetase remains constant after PBS 1 infection. The thyB coded pathway was also found to remain functional after PBS 1 infection. The "activity" as measured from the CPM released into the supernatant was lower in infected cells than in uninfected cells. The lower activity might be due to phosphorylation of the substrate for the thyB coded enzyme (5-^{3}H-dUMP) to dUDP by the PBS 1 induced dUMP kinase (59).

The synthesis of dUTP in PBS 1-infected cells.

The metabolism of pyrimidine compounds in B.subtilis and changes occurring during PBS 1 infection were investigated

in order to evaluate quantitatively the contribution of various pathways involved in the synthesis of dUTP in phage infected cells. The present study indicates that UTP reductase, dCTP deaminase and dCDP deaminase are involved in the synthesis of deoxyuridine nucleotides in PBS 1-infected cells. CdR deaminase and UdR kinase are not important in the synthesis of deoxyuridine nucleotides because UdR is converted rapidly to uracil and deoxyribose-1-phosphate. The PBS 1 induced dUMP kinase does probably not play an important role in the synthesis of dUDP or dUTP, since no biosynthetic pathways yielding dUMP in PBS 1-infected cells are present. The role of dUMP kinase is probably to block dephosphorylation of dUDP and dUTP so that these compounds can build up in PBS 1-infected cells. Another role of dUMP kinase may be the prevention of continued synthesis of thymidine nucleotides after PBS 1 infection (59). It removes the substrate for thymidylate synthetases by converting dUMP to dUDP.

In order to evaluate the contributions of the UTP reductase, dCTP deaminase and dCDP deaminase pathways, the ratio $dUMP^{x}/dCMP^{x}$ in PBS 1 DNA was measured in various strains of <u>B.subtilis</u> labelled with different radioactive bases and nucleosides. The validity of this measurement is dependent on the same four assumptions described in detail for the synthesis of dTTP in uninfected cells.

Matthews (85) showed that after T4 infection the pool sizes of the deoxyribonucleoside triphosphates did not change. The rate of DNA synthesis was much higher in T4-infected cells than in uninfected E.coli and it was assumed that the pools were maintained at a constant level by an even higher rate of turnover of the deoxyribonucleoside triphosphates after T4 infection (85). In PBS 1-infected B.subtilis, rates of DNA synthesis as measured by the rate of incorporation of radioactive CdR in infected cells were similar to that in uninfected cells (Table 28). The incorporation of radioactive CdR is rapid and this may indicate that the turnover of the dCTP pool is rapid in uninfected and PBS 1-infected cells of B.subtilis.

The assumption that deoxyribonucleoside triphosphates are the immediate precursors for PBS 1 DNA synthesis is supported by the requirement of DNA polymerase induced by PBS 1 for deoxyribonucleoside triphosphates in vitro (104). The deoxyribonucleoside diphosphates seem unlikely to be the precursors since the ratio $dUMP^{x}/dCMP^{x}$ in the DNA of PBS 1 labelled with 5-^{3}H-CdR is different from the ratio obtained with 5-^{3}H-CR. The presence of a dUMP kinase and dTMPase with dUMPase activity (59, 106) make it unlikely that deoxyribonucleoside monophosphates are the immediate precursors for PBS 1 DNA synthesis.

If the above assumptions are correct, the ratio $dUMP^{x}/dCMP^{x}$ would give an indication of the contribution of

the various pathways involved in the synthesis of dUTP in PBS 1-infected cells.

It appears that in a number of strains 50 % of the dUTP was derived from the reduction of UTP, and the remainder was derived from deamination of dCDP and dCTP. The differences in the ratios $dUMP^{x}/dCMP^{x}$ in PBS 1 DNA labelled with 5-^{3}H-CdR or with 5-^{3}H-CR suggested that dCDP deaminase must be also involved in the synthesis of dUTP in PBS 1-infected cells.

If we assume that 50 % of the dUTP in tsA13 cdd-1 is derived from the reduction of UTP (a similar proportion of dTTP was derived from the same source in uninfected cells), and if we assume further that the degree at which the dCTP and dCDP pools are diluted by unlabelled material remains constant after infection, then the parameter $(\frac{e}{c})$CdR / $(\frac{e}{c})$ CR does not change after infection and it is possible to estimate the contribution of the dCDP and dCTP deaminase pathways. It was shown that in PBS 1-infected cells the contribution of UTP reduction, dCTP deamination and dCDP deamination to the synthesis of dUTP was 50 %, 25 % and 25 % respectively. In ddd host strains which lacked dCDP deaminase, the lack of dCDP deaminase was compensated by an increased metabolic flux through the dCTP deaminase pathway.

S U M M A R Y

In B.subtilis, uracil and UR are converted to UMP. UdR is first degraded to uracil and deoxyribose-1-phosphate. UdR kinase is present in crude extracts of B.subtilis. However, this enzyme does not seem to be important in the metabolism of UdR.

Cytosine is not metabolized in B.subtilis. On the other hand, deoxycytidine-cytidine deaminase, deoxycytidine kinase and cytidine kinase, which concern the metabolism of cytosine nucleosides, are found. Mutants lacking these enzymes have been isolated.

Deaminase for cytidine ribonucleotides is absent in B.subtilis as in the case of enteric bacteria. Mutants blocked in the conversion of uridine nucleotides to cytidine nucleotides have been isolated in the present study. They are presumably blocked in CTP synthetase.

The observation of Wilson et al. (150) that two unlinked genetic loci, thyA and thyB, are involved in the synthesis of thymidine nucleotides in B.subtilis, has been confirmed. An enzyme activity which converts a deoxyuridine nucleotide to a thymidine nucleotide has been found. This enzyme is distinct from dTMP synthetase and dUMP is probably the substrate for this reaction.

A novel enzyme, dCDP deaminase, is found in B.subtilis. Properties of this enzyme are investigated and mutants lacking this enzyme have been isolated.

In B.subtilis, reduction of ribonucleotides appears to be taking place at the triphosphate level. Hydroxyurea inhibits the growth of B.subtilis and this inhibition can be overcome by deoxyribonucleosides. However, in vitro the reductase is insensitive to this drug.

Mutants which require deoxyribonucleosides for growth have been isolated. In these mutants, however, ribonucleotide reductase activity is still detectable.

The synthesis of dTTP in B.subtilis in PA has been investigated by in vivo labelling experiments. Under our conditions 17 % of dTTP is derived from exogenous TdR, 20 % is derived from exogenous CdR, 20 % is derived from reduction of CTP and the remainder (43 %) is derived from reduction of UTP.

As in the case of uninfected cells, UdR is not converted to dUMP in PBS 1-infected cells. When PBS 1 infects mutant cells lacking both CdR kinase and CdR deaminase, radioactive CdR is not recovered in nucleic acids. This suggests that neither CdR kinase nor CdR deaminase is induced by PBS 1.

The host enzyme, dCDP deaminase, remains active after PBS 1 infection. The enzyme activity coded by the thyB locus persists during the development of PBS 1. There is

no change in the level of ribonucleotide reductase in PBS 1-infected cells.

The pathways involved in the metabolism of pyrimidine compounds in PBS 1-infected cells are proposed on the bases of the present results and those of earlier studies on dTTPase (139), dTMPase (59, 106), dUMP kinase (59) and dCTP deaminase (188).

From the ratios of the specific activity of dUMP to that of dCMP in PBS 1 DNA labelled with radioactive bases or nucleosides in various mutants of B.subtilis, it is concluded that 50 % of dUTP is derived from reduction of UTP, 25 % is derived from deamination of dCDP and 25 % is derived from deamination of dCTP. In host mutants lacking dCDP deaminase, about 50 % of dUTP is derived from deamination of dCTP and the remainder is derived from reduction of UTP.

L I T E R A T U R E.

1. Alikhanian, S.I., T.S. Iljina, E.S. Kalieva, S.V. Kaneneva, and W. Sukhodolec. 1966. A genetical study of thymineless mutants of Escherichia coli K12. Genet. Res. 8: 83-100.

2. Anagnostopoulos, M.C., and A.M. Schneider. 1966. Déterminisme génétique de l'exigence en thymine chez certain mutants de Bacillus subtilis. Comptes Rendues Acad. Sciences. 262: 1311-1317.

3. Aposhian, H.V. 1965. A dTMPase found after infection of Bacillus subtilis with phage SP5C. Biochem. Biophys. Res. Commun. 18: 230-235.

4. Aposhian, H.V., and A. Kornberg. 1962. Enzymatic synthesis of DNA. The polymerase found after T2 infection. J. Biol. Chem. 237: 519-525.

5. Bagatell, F.K., E.M. Wright, and H.Z. Sable. 1959. Biosynthesis of ribose and deoxyribose in Escherichia coli. J. Biol. Chem. 234: 1369-1373.

6. Bariati, S. 1970. Incorporation of uridine into Bacillus subtilis and SPP1 bacteriophage Deoxyribonucleic acid. J. Bacteriol. 101: 330-332.

7. Barner, H.D., and S.S. Cohen. 1959. Virus induced acquisition of metabolic function. IV. Thymidylate synthetase in thymine requiring Escherichia coli infected by T2 and T5 bacteriophages. J. Biol. Chem. 234: 2987-2991.

8. Bazill, G.W., and D. Karamata. 1972. Temperature-sensitive mutants of Bacillus subtilis defective in deoxyribonucleotide synthesis. Mol. Gen. Genet. 117: 19-29.

9. Beck, C.F., J.L. Ingraham, J. Neuhard, and E. Thomassen. 1972. Metabolism of pyrimidines and pyrimidine nucleosides by Salmonella typhimurium. J. Bacteriol. 110: 219-228.

10. Beck,W.S.1967. Regulation of cobamide-dependent ribonucleotide reductase by allosteric effectors and divalent cations. J.Biol.Chem. 242: 3148-3158.

11. Beck,W.S., and M.Levin.1963. Purification, kinetics and repression control of bacterial trans-N-deoxyribosylase. J.Biol.Chem. 238: 702-709.

12. Beckman,L.D.,P.Witonsky, and D.J.McCorquodale.1972. Effect of rifampin on the growth of bacteriophage T5. J.Virol. 10: 179-186.

13. Belyeva,N.N., and R.R.Azizbekyan.1968. Fine structure of new Bacillus subtilis phage AR9 with complex morphology. Virology 34: 176-179.

14. Ben-Horin,H.,M.A.Waltz,and A.K.Saz.1974. Early biochemical events occurring after infection of Bacillus cereus 569SP1 with bacteriophage GSW. J.Virol. 13: 828-836.

15. Berglund,O.1972. Ribonucleoside diphosphate reductase induced by bacteriophage T4.I.Purification and characterisation. J.Biol.Chem. 247: 7270-7275.

16. Bertani,L.E.,A.Häggmark, and P.Reichard.1963. Enzymatic synthesis of deoxyribonucleotides.II.Formation and interconversion of deoxyuridine phosphates. J.Biol.Chem. 238: 3407-3413.

17. Billen,D.,L.Carreira,C.T.Hadden, and S.J.Silverstein. 1971. Evidence suggestive of compartmentalisation of deoxyribonucleic acid synthesizing systems in freeze-treated Bacillus subtilis. J.Bacteriol. 108: 1250-1256.

18. Billen,D.,L.Carreira, and S.J.Silverstein.1971. Utilisation of deoxyribonucleoside triphosphates in the cellular synthesis of DNA by Bacillus subtilis. Biochem.Biophys.Res.Commun. 43: 1150-1156.

19. Bodmer,W.F., and S.Grother.1965. Uptake and incorporation of thymine, thymidine, uracil, uridine, and 5-fluorouracil in the nucleic acids of Bacillus subtilis. J.Bacteriol. 89: 1011-1014.

20. Bradley,D.E.1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol.Rev. 31: 230-314.

21. Brockman, R.W., J.M.Davis, and P.Skitts.1960. Metabolism of uracil and 5-fluorouracil by drug-sensitive and by drug-resistant bacteria. Biochim.Biophys.Acta 40: 22-32.

22. Brodetsky, A.M., and W.R.Romig.1964. Characterisation of bacteriophages of Bacillus subtilis. Bact.Proc. 1964. p118.

23. Bujard, H.1969. Location of single stranded interruptions in the DNA of bacteriophage T5+. Proc.Natl. Acad.Sci.U.S. 62: 1167-1174.

24. Chace, K.V., and D.H.Hall.1973. Isolation of mutants of bacteriophage T4 unable to induce thymidine kinase activity. J.Virol. 12: 343-348.

25. Chamberlin, M., J.McGrath, and L.Waskell.1970. New RNA polymerase from Escherichia coli infected with bacteriophage T7. Nature 228: 227-231.

26. Clark, S., R.Losick, and J.Pero.1974. New RNA polymerase from Bacillus subtilis infected with phage PBS 2. Nature in press.

27. Cohen, S.S., J.G.Flaks, H.D.Barner, M.R.Loeb, and J.Lichtenstein.1958. The mode of action of 5-fluorouracil and its derivatives. Proc.Natl.Acad.Sci.U.S. 44: 1004-1012.

28. Davis, R.H.1963. Neurospora crassa mutant lacking an arginine specific carbamylphosphokinase. Science 142: 1652-1654.

29. Demerec, M., E.A.Adelberg, A.J.Clarke, and P.E.Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics 54: 61-76.

30. Dubnau, D.1970. Linkage map of Bacillus subtilis.p.I. 39-45. In.H.A.Sober and R.A.Harte (eds). Handbook of Biochemistry. (Second ed.) Chemical Rubber Co. Cleveland Ohio.

31. Eisenstark, A., R.Eisenstark, and S.Cunningham.1968. Genetic analysis of thymineless(thy) mutants in Salmonella typhimurium. Genetics 58: 493-506.

32. Eiserling, F.A.1967. The structure of Bacillus subtilis bacteriophage PBS 1. J.Ultrastruct.Res. 17: 342-347.

33. Elford, H. 1968. Effect of hydroxyurea on ribonucleotide reductase. Biochem. Biophys. Res. Commun. 33: 129-135.

34. Fangman, W.L., and A. Novick. 1966. Mutant bacteria showing efficient utilisation of thymidine. J. Bacteriol. 91: 2390-2394.

35. Fink, R.M., and K. Fink. 1961. Biosynthesis of radioactive RNA and DNA pyrimidines from thymidine-2-^{14}C. Biochem. Biophys. Res. Commun. 6: 7-10.

36. Flaks, J.G., and S.S. Cohen. 1959. Virus induced acquisition of metabolic function. I. Enzymatic formation of 5-hydroxymethyl-deoxycytidylate. J. Biol. Chem. 234: 1501-1506.

37. Flaks, J.G., J. Lichtenstein, and S.S. Cohen. 1959. Virus induced acquisition of metabolic function. II. Studies on the origins of the deoxycytidylate hydroxymethylase of bacteriophage infected Escherichia coli. J. Biol. Chem. 234: 1507-1511.

38. Frankel, R.W., and T.M. Joys. 1966. Adsorption specificity of bacteriophage PBS 1. J. Bacteriol. 92: 388-389.

39. Friedkin, M., and A. Kornberg. 1957. The enzymatic conversion of deoxyuridylic acid to thymidylic acid and the participation of tetrahydrofolic acid. p. 609-613. In W.D. McElroy and B. Glass (ed.) A symposium on the chemical basis of heredity. Johns Hopkins Press. Baltimore.

40. Fuchs, J.A., H.O. Karlström, H.R. Warner, and P. Reichard. 1972. Defective gene product in dnaF mutant of Escherichia coli. Nature New Biol. 238: 69-71.

41. Fuchs, J.A., and J. Neuhard. 1973. A mutant of Escherichia coli defective in ribonucleoside diphosphate reductase. Eur. J. Biochem. 32: 451-457-462.

42. Goldushek, E.P., and J.P. Sklar. 1969. Role of host RNA polymerase in phage development. Nature 221: 833-836.

43. Goulian, M., and W.S. Beck. 1966. Purification and properties of a cobamide dependent ribonucleotide reductase from Lactobacillus leichmannii. J. Biol. Chem. 241: 4233-4242.

44. Grass, K.B., and N.R. Cozzarelli. 1973. Further genetic and enzymological characterisation of the three Bacillus subtilis deoxyribonucleic acid polymerases. J.Biol.Chem. 248: 7688-7700.

45. Greenberg, G.R. 1966. New dUTPase and dUDPase activities after infection of Escherichia coli by T2 bacteriophage. Proc.Natl.Acad.Sci.U.S. 56: 1226-1232.

46. Greenberg. G.R., and R. Somerville. 1963. Deoxyuridylate kinase activity and deoxyuridine triphosphatase in Escherichia coli. Proc.Natl.Acad.Sci.U.S. 48: 247-257.

47. Grenson, M. 1969. The utilisation of exogenous pyrimidines and the recycling of uridine-5'-phosphate derivatives in Saccharomyces cerevisiae, as studied by means of mutants affected in pyrimidine uptake and metabolism. Eur.J.Biochem. 11: 249-260.

48. Hall, D.H. 1967. Mutants of bacteriophage T4 unable to induce dihydrofolate reductase activity. Proc.Natl. Acad.Sci.U.S. 58: 584-591.

49. Haselkorn, R., M. Vogel, and R.D. Brown. 1969. Conservation of the rifamycin sensitivity of transcription during T4 development. Nature 221: 836-838.

50. Haslam, E.A., D.H. Roscoe, and R.G. Tucker. 1967. Inhibition of thymidylate synthetase in bacteriophage infected Bacillus subtilis. Biochim.Biophys.Acta 134: 312-326.

51. Hemphil, H.E., H.R. Whiteley, L.R. Brown, and R.H. Doi. 1969. The effect of rifampin on the production of β-22 phage by Bacillus subtilis. Biochem.Biophys.Res.Commun. 37: 559-566.

52. Herrington, M.B. 1973. Ph.D. Thesis McMaster University. Temperature-sensitive mutants of bacteriophage PBS 2.

53. Herrington, M.B., and I. Takahashi. 1973. Mutagenesis of bacteriophage PBS 2. Mutation Res. 20: 275-278.

54. Hiraga, S., K. Igarashi, and T. Yura. 1967. A deoxythymidine kinase mutant of Escherichia coli. I. Isolation and some properties. Biochim.Biophys.Acta 145: 41-51.

55. Hunter,B.I.,H.Yamagishi, and I.Takahashi.1967. Molecular weight of bacteriophage PBS 1 deoxyribonucleic acid. J.Virol. 1: 841-842.

56. Ivanovics.G., and K.Csiszar.1962. Isolation and some characterisation of subtilis phages with transducing activity. Acta Microbiol.Acad.Sci.Hung. 9: 209-218.

57. Johnson,J.R., and D.H.Hall.1973. Isolation and characterisation of mutants of bacteriophage T4 resistant to folate analogues. Virology 53: 412-426.

58. Joys,T.M.1965. Correlation between susceptibility to bacteriophage PBS 1 and motility in Bacillus subtilis. J. Bacteriol. 90: 1575-1577.

59. Kahan,F.M.1963. Novel enzymes formed by Bacillus subtilis infected with bacteriophage. Fed.Proc. 22: 406.

60. Kahan,F.,E.Kahan, and B.Riddle.1964. Nucleotide metabolism in extracts of Bacillus subtilis infected with phage SP8. Fed.Proc. 23: 318.

61. Kallen,K.,M.Simon, and J.Marmur.1962. The occurrence of a new base replacing thymine in a bacteriophage DNA: 5-hydroxymethyluracil. J.Mol.Biol. 5: 248-250.

62. Kammen,H.O.1967. Thymine metabolism in Escherichia coli.I.Factors involved in utilisation of exogenous thymine. Biochim.Biophys.Acta. 134: 301-311.

63. Karlstrøm,H.O.1968. Mutants of Escherichia coli defective in ribonucleoside and deoxyribonucleoside catabolism. J.Bacteriol. 95: 1069-1077.

64. Karlstrøm.H.O.1970. Inability of Escherichia coli B to incorporate added deoxycytidine, deoxyadenosine and deoxyguanosine into DNA. Eur.J.Biochem. 17: 68-71.

65. Karlstrøm,H.O., and A.Larsson.1967. Significance of ribonucleotide reduction in the biosynthesis of deoxyribonucleotides in Escherichia coli. Eur.J. Biochem. 3: 164-170.

66. Kock,K.,H.R.Mahler, and D.Fraser.1960. Synthesis of deoxycytidine-5'-phosphate deaminase in Escherichia coli infected by T2 bacteriophage. Arch.Biochem. Biophys. 86: 85-88.

67. Kelln, R.A., and R.A.J.Warren.1973. Obligate thymidine auxotrophs of Pseudomonas acidovorans. J.Bacteriol. 113: 510-511.

68. Koerner, J.F., M.S.Smith, and J.M.Buchanan.1959. A deoxycytidine triphosphate splitting enzyme and the synthesis of deoxyribonucleic acid of T2 bacteriophage. J.Am.Chem.Soc. 81: 2594-2595.

69. Kornberg, A., S.B.Zimmerman, S.R.Kornberg, and J.Josse. 1959. Enzymatic synthesis of deoxyribonucleic acid. VI. Influence of bacteriophage T2 on the synthetic pathway in host cells. Proc.Natl.Acad.Sci.U.S. 45: 772-785.

70. Krupinski, A.M., R.J.Bose, and R.A.J.Warren.1973. 5-(4-aminobutyl aminomethyl)-uracil, an unusual pyrimidine from the DNA of bacteriophage φ-W-14. Biochemistry 12: 151-157.

71. Langridge, R., and J.Marmur.1964. X-ray diffraction study of a DNA which contains uracil. Science 143: 1450-1451.

72. Larsson, A., and P.Reichard.1966. Enzymatic synthesis of deoxyribonucleotides.X.Reduction of purine ribonucleotides; allosteric behaviour and substrate specificity of the enzyme system from Escherichia coli B. J.Biol.Chem. 241: 2540-2549.

73. Larsson, A., and P.Reichard.1967. Enzymatic reduction of ribonucleotides. Progress Nucl.Acid Res. 7: 303-347.

74. Laurent, T.C., E.C.Moorr, and P.Reichard.1964. Enzymatic synthesis of deoxyribonucleotides.IV.Isolation and characterisation of thioredoxin, the hydrogen donor from Escherichia coli B. J.Biol.Chem. 239: 3436-3444.

75. Lavi, U., and M.Marcus.1972. Arrest of host DNA synthesis in Bacillus subtilis infected with phage φ∈ Virology 49: 668-674.

76. Lederberg.J. and E.M.Lederberg.1952. Replica plating and indirect selection of mutants. J.Bacteriol. 63: 399-406.

77. Lomax, M.I.S., and G.R.Greenberg.1967. A new assay of thymidylate synthetase based on the release of tritium from deoxyuridylate-5-^{3}H. J.Biol.Chem. 242: 109-113.

78. Lovett, P.S. 1972. PBP 1. A flagella-specific bacteriophage mediating transduction in Bacillus pumilus. Virology 47: 743-752.

79. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randell. 1951. Protein measurement with the folin phenol reagent. J.Biol.Chem. 193: 265-275.

80. Lozeron, H.A., and W. Szybalski. 1967. Incorporation of 5-fluoro-deoxyuridine into the DNA of Bacillus subtilis phage PBS 2 and its radiobiological consequences. J.Mol.Biol. 30: 277-290.

81. Mahler, I. 1968. Procedures for Bacillus subtilis transformation. p.846. In L.Grossman, and K.Moldave (Eds.). Methods of Enzymology, XII B. Academic Press Inc., New York.

82. Mahler, I., M.Cahoon, and J.Marmur. 1964. Bacillus subtilis DNA transfer in PBS 2 transduction. J.Bacteriol. 87: 1423-1428.

83. Maley, F, G.F. Maley, and J.F. McGarrahan. 1967. A simplified enzymatic procedure for the preparation of nucleoside di- and triphosphates. Anal.Biochem. 19: 265-271.

84. Marmur. J. et al. 1972. Unique properties of nucleic acid from Bacillus subtilis phage SP15. Nature New Biol. 239: 68-70.

85. Matthews, C.K. 1972. Biochemistry of deoxyribonucleic acid defective amber mutant of bacteriophage T4. III. Nucleotide pools. J.Biol.Chem. 247: 7430-7438.

86. Milanesi, G., and G.Cassani. 1972. Transcription after bacteriophage SPP 1 infection in Bacillus subtilis. J.Virol. 10: 187-192.

87. Munch-Peterson, A., and M.Schwartz. 1972. Inhibition of the catabolism of deoxyribonucleosides in Escherichia coli after infection by T4 phage. Eur. J.Biochem. 27: 443-447.

88. Myers, D.E., and A.Landy. 1973. The role of host RNA polymerase in P1 phage development. Virology 51: 521-524.

89. Neubert, S., and J.Marmur. 1973. Synthesis of the unusual DNA of Bacillus subtilis bacteriophage SP15. J.Virol. 12: 1078-1084.

90. Neuhard, J. 1968. Pyrimidine nucleoside metabolism and pathways of thymidine triphosphate biosynthesis in Salmonella typhimurium. J.Bacteriol. 96: 1519-1527.

91. Neuhard, J., and J.Ingraham. 1968. Mutants of Salmonella typhimurium requiring cytidine for growth. J.Bacteriol. 95: 2431-2433.

92. Neuhard, J., and E.Thomassen. 1971. Turnover of the deoxyribonucleoside triphosphates in Escherichia coli 15 T during thymine starvation. Eur.J.Biochem. 20: 36-43.

93. Neuhard, J., and E.Thomassen. 1971. Deoxycytidine triphosphate deaminase: identification and function in Salmonella typhimurium. J.Bacteriol. 105: 657-665.

94. Nierlich, D.P. 1967. Radioisotope uptake as a measure of synthesis of messenger RNA. Science 158: 1186-1188.

95. Nishihara, M., A.Cramback, and H.V.Aposhian. 1967. The deoxycytidylate deaminase found in Bacillus subtilis infected with SP82. Biochemistry 6: 1877-1886.

96. O'Donovan, G.A., G.Edlin, J.A.Fuchs, J.Neuhard, and E.Thomassen. 1971. Deoxycytidine triphosphate deaminase: characterisation of an Escherichia coli mutant deficient in the enzyme. J.Bacteriol. 105: 666-672.

97. O'Donovan, G.A., and J.Neuhard. 1970. Pyrimidine metabolism in microorganisms. Bacteriol.Rev. 34: 278-343.

98. Okazaki, T., and A.Kornberg. 1964. Enzymatic synthesis of nucleic acid. XV. Purification and properties of a polymerase from Bacillus subtilis. J.Biol.Chem. 239: 259-268.

99. Okubo, S., B.Strauss, and M.Stodolsky. 1964. The possible role of recombination in the infection of competent Bacillus subtilis by bacteriophage deoxyribonucleic acid. Virology 24: 552-562.

100. Øvrebo, S., and K.Kleppe. 1973. Pyrimidine metabolism in Acinetobacter calcoaceticus. J.Bacteriol. 116: 331-336.

101. Palacek, E. 1965. Fate of Bacillus subtilis DNA after phage infection. Folia Biologica (Praha) 11: 89-95.

102. Péne, J.J. and J.Marmur.1964. Infectious DNA from a virulent bacteriophage active on transformable Bacillus subtilis. Fed. Proc.23: 318.

103. Peterson, R.N., J.Boniface, and A.L.Koch.1967. Energy requirements, interactions and distinction in the mechanism of transport of various nucleosides in Escherichia coli. Biochim. Biophys. Acta 135: 771-783.

104. Price, A.R., and S.J.Cook.1972. New deoxyribonucleic acid polymerase induced by Bacillus subtilis bacteriophage PBS 2. J.Virol. 9: 602-610.

105. Price, A.R., L.F.Dunham, and R.L.Walker.1972. Thymidine triphosphate nucleotidohydrolase and deoxyuridylate hydroxymethylase induced by mutants of Bacillus subtilis bacteriophage SP82G. J.Virol. 10: 1240-1241.

106. Price, A.R., and S.M.Fogt.1973. Deoxythymidylate phosphohydrolase induced by bacteriophage PBS 2 during infection of Bacillus subtilis. J.Biol. Chem. 248: 1372-1380.

107. Price, A.R., and S.M.Fogt.1973. Resistance of bacteriophage PBS 2 infection to 6-(p-hydroxyphenylazo-)-uracil, an inhibitor of Bacillus subtilis deoxyribonucleic acid synthesis. J.Virol. 11: 338-340.

108. Price, A.R., and S.M.Fogt.1973. Effect of naladixic acid on PBS 2 bacteriophage infection of Bacillus subtilis. J.Virol. 12: 405-407.

109. Price, A.R., and M.Frabotta.1972. Resistance of bacteriophage PBS 2 infection to rifampicin, an inhibitor of Bacillis subtilis RNA synthesis. Biochem.Biophys.Res.Commun. 48: 1578-1585.

110. Price, A.R., R.Hitzeman, J.Frato, and K.Lombardi.1974. Rifamycin resistant bacteriophage PBS 2 infection and RNA polymerase. In preparation.

111. Pritikin, W.B., and H.Reiter.1969. Abortive infection of Bacillus subtilis bacteriophage PBS 1 in the presence of actinomycin D. J.Virol. 3: 578-585.

112. Raimondo, L.M., N.P.Lundh, and R.J.Martinez.1968. Primary adsorption site of phage PBS 1: the flagellum of Bacillus subtilis. J.Virol. 2: 256-264.

113. Reichard, P., A. Baldestec, and L. Rutberg. 1960. Formation of deoxycytidine phosphates from cytidine phosphates in extracts from Escherichia coli. J. Biol. Chem. 236: 1150-1157.

114. Rima, B.K., and B.H.A. van Kleeff. 1971. Similarity of Bacillus subtilis bacteriophages PBS 1, 3NT and I10. Anth. v. Leeuwenhoek. 37: 265-274.

115. Rima, B.K., and I. Takahashi. 1973. The synthesis of nucleic acids in Bacillus subtilis infected with phage PBS 1. Can. J. Biochem. 51: 1219-1224.

116. Rinehart, K.V., and J.C. Copeland. 1973. Evidence that thymine is not a normal metabolite in wild type Bacillus subtilis. Biochim. Biophys. Acta 294: 1-7.

117. Roscoe, D.H. 1969. Thymidine triphosphate nucleotidohydrolase: a phage induced enzyme in Bacillus subtilis. Virology 38: 520-526.

118. Roscoe, D.H., and R.G. Tucker. 1966. The biosynthesis of 5-hydroxymethyluridylic acid in bacteriophage infected Bacillus subtilis. Virology 29: 157-166.

119. Sanderson, K.E. 1970. Current linkage map of Salmonella typhimurium. Bacteriol. Rev. 34: 176-193.

120. Schachtele, C.F., C.V. deSain, L.A. Hawley, and D.L. Anderson. 1972. Transcription during the development of bacteriophage φ-29: Production of host- and φ-29 specific ribonucleic acid. J. Virol. 10: 1170-1178.

121. Siedler, A.J., and M.T. Holtz. 1963. Regulatory mechanisms in the deoxyribonucleic acid metabolism of Lactobacillus acidophilus R-26. Deoxycytidylate deaminase activity. J. Biol. Chem. 238: 697-701.

122. Sinha, N.K., and D.P. Snustad. 1972. Mechanism of inhibition of deoxyribonucleic acid synthesis in Escherichia coli by hydroxyurea. J. Bacteriol. 112: 1321-1334.

123. Sjöberg, B.M. 1972. Studies on the structure of T4 thioredoxin: I. Isolation and amino acid sequence of the peptides obtained from a tryptic digest of the protein. J. Biol. Chem. 247: 8058-8062.

124. Sjöberg.B.M., and A.Holmgren.1972. Studies on the structure of T4 thioredoxin.II.Amino acid sequence of the protein and comparison with thioredoxin from Escherichia coli. J.Biol.Chem. 247: 8063-8068.

125. Somerville.R.,K.Ebisuzaki, and G.R.Greenberg.1959. Hydroxymethyldeoxycytidylate kinase formation after bacteriophage infection of Escherichia coli. Proc.Natl.Acad.Sci.U.S. 45: 1240-1245.

126. Spizizen.J.1958. Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleate. Proc.Natl.Acad.Sci.J.S. 44: 1072-1078.

127. Striniste,G.F., and W.D.Taylor.1972. Development of bacteriophage α.II. Dependence on the host RNA polymerase. J.Virol. 10: 1031-1036.

128. Takahashi,I.1961. Genetic transduction in Bacillus subtilis. Biochem.Biophys.Res.Commun. 5: 171-175.

129. Takahashi,I.1963. Transducing phages for Bacillus subtilis. J.gen.Microbiol. 31: 211-217.

130. Takahashi,I.1964. Incorporation of bacteriophage genome by spores of Bacillus subtilis. J.Bacteriol. 87: 1499-1502.

131. Takahashi,I.1965. Localisation of spore markers on the chromosome of Bacillus subtilis. J.Bacteriol. 89: 1065-1067.

132. Takahashi,I., and J.Marmur.1963. Replacement of thymidylic acid by deoxyuridylic acid in the deoxyribonucleic acid of a transducing phage for Bacillus subtilis. Nature 197: 794-795.

133. Taylor,A.L.1970. Current linkage map of Escherichia coli. Bacteriol. Rev. 34: 155-175.

134. Taylor,M.J., and C.B.Thorne.1963. Transduction of Bacillus licheniformis and Bacillus subtilis by each of two phages. J.Bacteriol. 86: 452-461.

135. Thelander,L.1973. Physicochemical characterisation of ribonucleoside diphosphate reductase from Escherichia coli. J.Biol.Chem. 248: 4591-4601.

136. Thorne,C.B.1962. Transduction in Bacillus subtilis. J.Bacteriol. 83: 106-111.

137. Tomita, F. 1968. Ph.D. Thesis. McMaster University. Biochemical Studies on Bacteriophage PBS 1.

138. Tomita, F., and I. Takahashi. 1969. A novel enzyme dCTP deaminase, found in Bacillus subtilis infected with phage PBS 1. Biochim. Biophys. Acta 179: 18-27.

139. Tomita, F., and I. Takahashi. 1974. Enzymes concerning the biosynthesis of uracil containing DNA. In preparation.

140. Tyeryar, F. J., M. J. Taylor, W. D. Lawton, and I. D. Goldberg. 1969. Cotransduction and cotransformation of genetic markers in Bacillus subtilis and Bacillus licheniformis. J. Bacteriol. 100: 1027-1036.

141. Vanyushin, B. F. et al. 1969. Some characteristics of uracil containing DNA from AR9 phage for Bacillus subtilis. Molekularjana Biologija 4: 724-729

142. Wachsman, J. 1973. Demonstration of a CdR kinase activity in extracts of Bacillus megaterium. Appl. Microbiol. 25: 506-509.

143. Wang, T. P., and J. O. Lampen. 1951. The cleavage of adenosine, cytidine and xanthosine by Lactobacillus pentosus. J. Biol. Chem. 192: 339-347.

144. Warner, H. R., and M. D. Hobbs. 1969. Effect of hydroxyurea on replication of bacteriophage T4 in Escherichia coli. J. Virol. 3: 331-336.

145. Warner, H. R., D. P. Snustadt, S. E. Jorgensen, and J. F. Koerner. 1970. Isolation of bacteriophage T4 mutants defective in the ability to degrade host deoxyribonucleic acid. J. Virol. 5: 700-708.

146. Werner, R. 1971. Nature of DNA precursors. Nature New Biol. 233: 99-103.

147. Wahba, A. J., and M. Friedkin. 1962. The enzymatic synthesis of thymidylate. I. Early steps in the purification of thymidylate synthetase from Escherichia coli. J. Biol. Chem. 237: 3794-3801.

148. Wickner, W., and A. Kornberg. 1973. DNA polymerase III requires ATP to start synthesis on a primed DNA. Proc. Natl. Acad. Sci. U.S. 70: 3679-3683.

149. Wickner,W.,R.Schekman,K.Geider, and A.Kornberg.1973. A new form of DNA polymerase III and a copolymerase replicate a long single-stranded primer template. Proc.Natl.Acad.Sci.U.S. 70: 1764-1767.

150. Wilson,M.C.,J.L.Farmer, and F.Rothman.1966. Thymidylate synthesis and aminopterin resistance in Bacillus subtilis. J.Bacteriol. 92: 186-196.

151. Wovcha,M.G.,P.K.Tomlich,C.S.Chiu, and G.R.Greenberg. 1973. Direct participation of dCMP hydroxymethylase in the synthesis of bacteriophage T4 DNA. Proc. Natl.Acad.Sci.U.S. 70: 2196-2200.

152. Wright,B.E.1973. Critical variables in differentiation.pp1-109. 8-25. Prentice-Hall,Inc. Englewood Cliffs. New Jersey.

153. Wu,R., and A.D.Kaiser.1967. Mapping of the 5'-terminal nucleotides of the DNA of bacteriophage λ and related phages. Proc.Natl.Acad.Sci.U.S. 57: 170-177.

154. Wyatt,G.R., and S.S.Cohen.1953. The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-hydroxymethylcytosine. Biochem.J. 55: 774.

155. Yamagishi,H.1968. Single strand interruptions in PBS 1 bacteriophage DNA molecule. J.Mol.Biol. 35: 623-633.

156. Yamagishi,H., and I.Takahashi.1968. Transducing particles of PBS 1. Virology 36: 639-645.

157. Yeh,Y-C.,E.J.Dubovi, and I.Tessman.1969. Control of pyrimidine biosynthesis by bacteriophage T4, mutants unable to catalyze the reduction of cytidine diphosphate. Virology 37: 615-623.

158. Yehle,Co., and A.T.Ganesan. Deoxyribonucleic acid synthesis in bacteriophage SP01 infected Bacillus subtilis. J.Virol. 9: 263-272.