MECHANISMS OF THYMINE INCORPORATION IN *ESCHERICHIA COLI* AND *SALMONELLA TYPHIMURIUM*

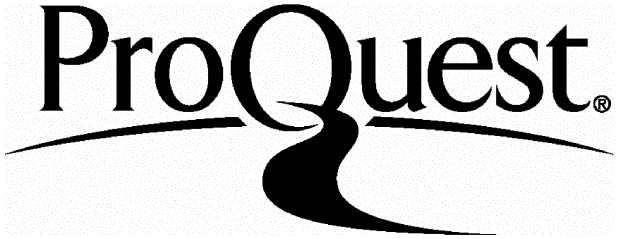
Thesis submitted for the Degree of Doctor of Philosophy

at the University of Leicester

1970

by

I.R. Beacham, B.Sc.



The Road goes ever on and on

Down from the door where it began.

Now far ahead the Road has gone,

And I must follow, if I can,

Pursuing it with eager feet,

Until it joins some larger way

Where many paths and errands meet.

And whither then? I cannot say.

J.R.R. Tolkien.

INDEX

INTRODUCTION

Escherichia coli and *Salmonella typhimurium* are able to incorporate most bases added to the growth medium into their nucleic acids. Uracil, for example, is taken up *via* uridine-5'-monophosphate pyrophosphorylase, viz. uracil + 5' phosphoribosyl-1-pyrophosphate ⟶ UMP + pyrophosphate (Crawford *et al*, 1957; Molloy & Finch, 1969). Other bases are also incorporated, almost certainly by a similar type of reaction (see Magasanik, 1962), although the enzymes concerned have not been characterised. Such reactions are not involved in the *de novo* synthesis of purines and pyrimidines, but presumably function to 'salvage' bases in the medium. The efficiency of salvage pathways in the metabolic economy of the cell must clearly depend upon feedback inhibition of the *de novo* synthetic route, or no economy would result.

This situation does not apply, however, to the incorporation of exogenous thymine into DNA. Wild-type strains of *E.coli* are totally unable to incorporate thymine added to the medium into DNA-thymine even when high concentrations (100-400 μM) are used (Bolton & Reynard, 1954; Crawford, 1958; Boyce & Setlow, 1962; Rachmeler *et al*, 1961). This is unlikely to be due to lack of permeability to thymine since it has been shown that treatment

with EDTA, which increases the permeability of the cell membrane (Lieve, 1968), does not permit incorporation of thymine into DNA (Munch-Petersen, 1967; Kammen, 1967a). Also, the incorporation of thymine into the DNA of wild-type E.coli can be potentiated by the addition of deoxynucleosides to the medium (Boyce & Setlow, 1962; Kammen, 1967b; Munch-Petersen, 1967). Thus no salvage pathway exists for the incorporation of thymine into DNA.

In contrast to thymine, thymidine is readily taken up and incorporated by wild-type E.coli and Salmonella typhimurium. The incorporation, however, lasts only a short time due to rapid breakdown of thymidine to thymine by thymidine phosphorylase. Mutants which have lost thymidine phosphorylase activity incorporate thymidine indefinitely.

Although wild-type strains of E.coli are unable to incorporate external thymine, mutants can be isolated, using aminopterin or trimethoprim as selective agents, which require thymine for growth (Okada et al, 1961; Stacey & Simpson, 1965). Such mutants, termed "thymine-less" mutants are the result of a single mutational lesion located at 55 min on the E.coli genetic map (Alikhanian et al, 1966). Thymine-requiring mutants possess the ability to incorporate external thymine although high concentrations (200 μM) are needed. Thus they are pleiotropic: they have lost the ability to synthesise

thymidylate de novo and have gained the ability to utilise thymine added to the growth medium.

Thy$^-$ mutants, as isolated by aminopterin or trimethoprim selection, have been shown to lack thymidylate synthetase (Barner & Cohen, 1951; Mantsavinos & Zamenhof, 1961; Breitman & Bradford, 1967). They are designated thy$^-$ (see Taylor & ~~Thoman~~ Trotter, 1967). Thymidylate synthetase catalyses the methylation of dUMP to TMP, and, until recently, has been assumed to be on the normal de novo route for the synthesis of thymidylate. However, it has quite recently been found that approximately 80% of the thymine in DNA is derived from cytosine compounds, rather than from uracil compounds (Karlstrom & Larsson, 1967; Neuhard, 1968). Förster and Holldorf have isolated from E.coli two enzymes capable of converting dCTP to TTP, viz. (1) dCTP + 5, 10 methylene tetrahydrofolate $\longrightarrow$ 5-methyl dCTP + dihydrofolate; (2) 5-methyl dCTP $\longrightarrow$ TTP + NH_3 (Förster & Holldorf, 1965). Thus there are two different pathways for the synthesis of dTTP in E.coli, and the precise nature of the defect in thy$^-$ mutants must therefore be in doubt at the present time.

Two routes for the uptake of thymine in thy$^-$ mutants also seem possible. The first is via a deoxyribosyl transferase reaction, viz. deoxynucleoside (XdR) + thymine (T) $\longleftrightarrow$ thymidine (TdR) + base (X). The second is via thymidine phosphorylase, viz. deoxyribose-1-phosphate (dRib-1-P) + thymine $\longleftrightarrow$ thymidine + phos-

phate (see Fig. 1). There is evidence, which will be discussed later, to show that the second route is the one normally used. Thus an adequate supply of a deoxyribosyl donor (which in fact is dRib-1-P) is clearly necessary for the utilisation of external thymine. It seems possible that in wild-type strains of E.coli this compound is not formed in sufficient quantity to permit the synthesis of significant amounts of thymidylate from externally supplied thymine. If in thy$^-$ mutants dRib-1-P is formed, the problem arises as to how and by what route it is synthesised.

The first thymineless mutants were isolated by Roepke (1947; 1967) and were induced with X-rays. Such mutants readily gave rise to secondary mutants capable of growing on less than one-tenth the amount of thymine required by the original mutant (Roepke, 1967). This has also been reported by Harrison (1965) to occur with thy$^-$ mutants of E.coli and Aerobacter aerogenes. Clearly such mutants might be useful in elucidating the pleiotropic nature of thy$^-$ mutants.

The secondary mutants require only about 20 μM thymine in the growth medium for normal growth, and map close and to the left side of the tyr locus (Alikhanian et al, 1966; Okada, 1966). This is distinct from the thy$^-$ locus at 55 min on the E.coli genetic map (Alikhanian et al, 1966; Taylor & Trotter, 1967).

Fig. 1. Pathways of Deoxynucleoside Catabolism

1 = Thymidine phosphorylase

2 = Purine nucleoside phosphorylase

3 = Phosphodeoxyribomutase

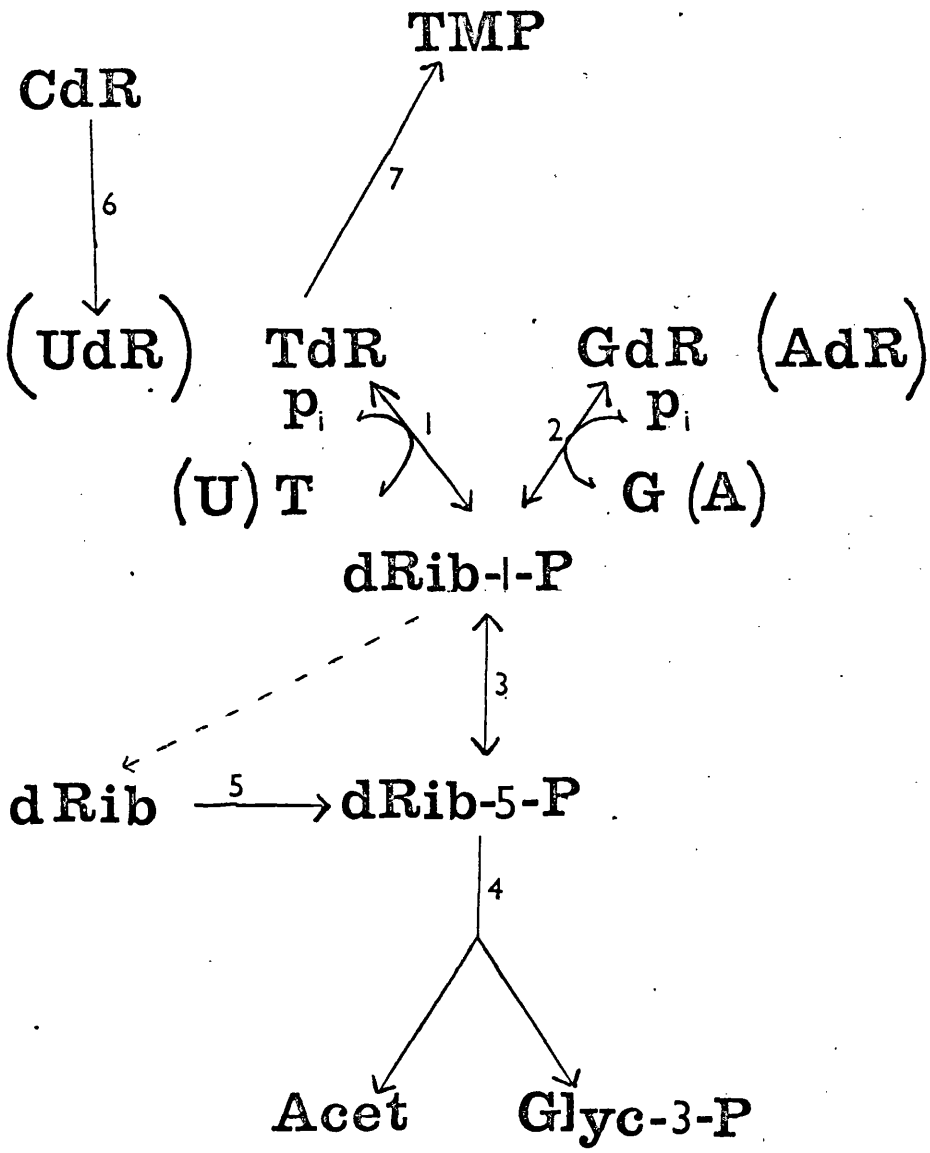
4 = Deoxyriboaldolase

5 = Deoxyribokinase

6 = Cytidine deaminase

7 = Thymidine kinase. This is an anabolic enzyme.

(For references to these enzymes, see legend to chart 1.)


TMP
CdR
6
7
(UdR)
TdR
GdR
(AdR)
Pi
Pi
1
2
(U) T
G (A)
dRib-1-P
3
dRib
5
dRib-5-P
4
Acet
Glyc-3-P

The second mutation has been designated *tlr* (thymine low requiring) by Alikhanian *et al* (1966).

In comparison with *thy*⁻ mutants, *thy*⁻ *tlr*⁻ strains are known to have three distinguishing properties, apart from their ability to grow on low concentrations of thymine. They excrete deoxyribose into the medium, thymidine phosphorylase is induced when the culture is deprived of thymine (Breitman & Bradford, 1964; Smith, 1964), and, in some *tlr* mutants, growth is impaired by the presence of thymidine in the medium (Alikhanian *et al*, 1966). Alikhanian *et al* showed that the mutation responsible for this thymidine sensitivity is linked to the *tlr* locus.

The behaviour of *tlr* mutants when thymine starved, together with the observation that addition of deoxynucleosides to the medium allows the utilisation of thymine in wild-type strains, led to the hypothesis that the production of deoxyribose derivatives (deoxynucleotides and deoxynucleosides) is greater in *tlr* mutants (Mantsavinos & Zamenhof, 1961; Breitman & Bradford, 1964; Munch-Petersen, 1967). Munch-Petersen (1967) suggested that a larger net production of deoxynucleosides provided the deoxyribosyl groups necessary for uptake of thymine via a deoxyribosyl transferase reaction.

A recent breakthrough in the understanding of *thy*⁻ *tlr*⁻

mutants was made by Breitman and Bradford (1967). They showed that one tlr mutant lacked deoxyriboaldolase activity. From the known pathways of deoxynucleoside catabolism (see Fig.1) it was therefore postulated that the block in catabolism of deoxyribose-5-phosphate results in an enhanced pool size of deoxyribose-1-phosphate which then allows efficient utilisation of thymine via thymidine phosphorylase. The low thymine requirement of thy$^-$ tlr$^-$ strains was thus seen to be a result of a block in the catabolism of deoxyribose phosphates rather than an increased rate of synthesis of these compounds.

The work reported in this thesis was directed towards a greater understanding of spontaneous tlr mutants, and their properties, using biochemical and genetic methods.

An understanding of such mutants is of technical importance with respect to the use of radioactive thymine and thymidine in measurements of DNA synthesis. It is also considered of importance in understanding the factors involved in the control of DNA synthesis.

ABBREVIATIONS

A = adenine; G = guanine; HX = hypoxanthine; U = uracil; T = thymine; C = cytosine; Rib = ribose; dRib = deoxyribose; Rib-1-P = ribose-1-phosphate; Rib-5-P = ribose-5-phosphate; dRib-1-P = deoxyribose-1-phosphate; dRib-5-P = deoxyribose-5-phosphate; AR = adenosine; GR = guanosine; UR = uridine; TR = ribosyl thymine; CR = cytidine; AdR = deoxyadenosine; GdR = deoxyguanosine; HXdR = deoxyinosine; UdR = deoxyuridine; TdR = thymidine; CdR = deoxycytidine; GMP, GDP, GTP = guanosine mono, di and tri-phosphate respectively; AMP, ADP, ATP = adenosine mono, di and triphosphate respectively; XMP = xanthine monophosphate; IMP = inosine monophosphate; UMP, UDP, UTP = uridine mono, di and triphosphate; CMP, CDP and CTP = cytidine mono, di and triphosphate respectively; dAMP, dADP, dATP = deoxyadenosine mono, di and triphosphate respectively; dGMP, dGDP, dGTP = deoxyguanosine mono, di and triphosphate respectively; dUMP, dUDP, dUTP = deoxyuridine mono, di and triphosphate respectively; dCMP, dCDP, dCTP = deoxycytidine mono, di and triphosphate respectively; TMP, TDP, TTP = thymidine mono, di and triphosphate respectively; PRPP = 5'-phosphoribosyl-1-pyrophosphate; 5-medCTP = 5-methyl-deoxycytidine triphosphate; P_i = orthophosphate; pp_i = pyrophosphate; Glyc-3-P = glyceraldehyde-3-phosphate; Acet = acetaldehyde. Phosphodeoxyribomutase and deoxy-

riboaldolase are abbreviated to mutase and aldolase respectively. The following gene symbols are used:

thy = thymine requirement; tlr low thymine requirement;

dra = deoxyriboaldolase; drm = phosphodeoxyribomutase;

tpp = thymidine phosphorylase; pup = purine nucleoside phosphorylase;

cdd = cytidine deaminase; thr = threonine; leu = leucine;

thi = thiamine; gal = galactose.

MATERIALS

All materials were obtained from commercial sources, mainly from Sigma. Fluorodeoxycytidine was a gift from Roche Products Ltd. Hydroxyurea was obtained from E.R. Squibb & Son. Trimethoprim was obtained from Burroughs Wellcome & Co. Xanthine oxidase was obtained from Worthington Biochemical Corporation.

METHODS

Bacteria. A list of strains used is shown in Table 1.

Cultural Conditions. Unless otherwise stated, all experiments were performed in M9 synthetic medium (Anderson, 1946) which was solidified with agar (1.5%) when required. Cultures were aerated by shaking in a New Brunswick gyrotory shaker.

Isotope Incorporation. Incorporation of ^{14}C and ^{3}H thymine into DNA was determined by pipetting 0.05 ml samples onto Whatman No.1 filter discs which were dropped immediately into ice cold TCA (5%),

Strain 2006 and its derivatives are Salmonella typhimurium KSU (LT2). All other strains are Escherichia coli K12. Strain NZ was kindly donated by Professor P. Starlinger.

TABLE 1. List of Bacterial Strains

Strain	Genotype	Additional requirements	Derivation
2006	thy$^-$ tlr$^+$		
2454	thy$^-$ dra$^-$	thr$^-$	From 2006
2456	thy$^+$ dra$^-$	thr$^-$	From 2454
2793	thy$^+$ tlr$^+$		
C600	thy$^+$ tlr$^+$	thr$^-$ leu$^-$ thi$^-$	
P152	thy$^-$ drm$^-$	leu$^-$ thi$^-$	
CR34	thy$^-$ dra$^-$	thr$^-$ leu$^-$ thi$^-$	
162-8	thy$^-$ dra$^-$ drm$^-$		From CR34
162-19	thy$^-$ dra$^-$ tpp$^-$		From CR34
162-B4	thy$^-$ dra$^-$ pup$^-$		From CR34
162-19A17	thy$^-$ dra$^-$ tpp$^-$ pup$^-$		From 162-19
162-B41	thy$^-$ dra$^-$ tpp$^-$ pup$^-$		From 162-B4
162-14	thy$^-$ dra$^-$ tpp$^-$ pup$^-$		From CR34
W945	thy$^+$ tlr$^+$		
W945-3	thy$^-$ tlr$^+$		From W945
W945-15	thy$^-$ drm$^-$		From W945-3
W945-10	thy$^-$ dra$^-$		From W945-3
W945-10R	thy$^+$ dra$^-$		From W945-10
NZ	thy$^+$ thr$^+$	gal$^-$	

washed three times in batches in ice cold TCA (5%), once in ethanol and once in ether, air dried, and counted in a Packard Scintillation counter. ^{32}P incorporation into DNA and RNA was measured by the method of Roodyn and Mandel (1960) using Millipore filters (0.45 μ). The ^{32}P was hydrolysed in 1 N HCl at 100°C for 30 min before use (Kjeldgaard, 1961).

Preparation of Cell Extracts. For the estimation of transketolase-transaldolase activity, cells were harvested by centrifugation, washed and resuspended in Tris (5 mM) -$MgCl_2$ (1 mM) - HCl buffer (pH 7.4) at approximately 2 x 10^{10} cells/ml and disrupted at 0°C with an ultrasonicator. The extract was spun at 20,000 g for 10-15 min at 0-4°C before use. For the assay of most other enzymes, cells were resuspended in Tris (0.1 M) -HCl buffer (pH 7.4) at a concentration of about 5 x 10^9 cells/ml and treated similarly.

Osmotic shock was performed as described by Neu and Heppel (1964) on 5 ml or 2.5 ml of cells at 10^{10}/ml.

Enzyme assays. Thymidine phosphorylase was assayed by determining the rate of the formation of thymine from thymidine. The assay mixture contained 5 mM thymidine, 10 mM phosphate buffer (pH 6.5), and the appropriate amount of enzyme in a total volume of 0.5 ml. Samples were removed and thymine measured at pH 13 at 300 mμ (Hotchkiss, 1948). A change in molar extinction coefficient, for the conversion of thymidine to thymine, of $4.3 \times 10^{+3}$ was used.

Deoxyriboaldolase was measured in two ways: (1) by measuring the disappearance of diphenylamine reacting material (Burton,1956). The reaction mixture contained 20 mM dRib-5-P and 50 mM Tris-maleate buffer (pH 6.9) in a total volume of 0.5 ml. (2) The reaction was coupled to the oxidation of NADH to NAD via alcohol dehydrogenase and both triosephosphate isomerase and L-α-glycerol dehydrogenase (Racker, 1952; Hoffee, 1968). The reaction mixture contained 10 µg of alcohol dehydrogenase, 1 µl of triose phosphate isomerase, 10 µl of L-α-glycerol dehydrogenase, 0.1 mg (14 mµmoles) of $NADH_2$, 2 mM dRib-5-P, and 50 mM Tris-HCl buffer (pH 7.5) in a total volume of 1.0 ml. The reaction was followed at 340 mµ.

Purine nucleoside phosphorylase was determined by measuring the formation of hypoxanthine from inosine after conversion of the former to uric acid in the presence of excess xanthine oxidase. The increase in optical density at 290 mµ was followed (Kalckar, 1947; Gardner & Kornberg, 1967). The reaction mixture contained 40 mM phosphate buffer (pH 7.1), 10 µl (0.03 units; Gardner & Kornberg, 1967) of xanthine oxidase, 200 mM sodium acetate buffer (pH 5.9) and 2 mM inosine. A change in molar extinction coefficient, for the conversion of inosine to uric acid, of 10.8×10^3 was used.

Phosphodeoxyribomutase was measured in two ways: (1) by measuring

the disappearance of dRib-1-P and formation of dRib-5-P. In cell extracts from deoxyriboaldolase positive strains, very little dRib-5-P formation is observed. The reaction mixture of 0.5 ml contains 10 mM NaF and 1 mM dRib-1-P. Samples (0.5 ml) were taken into an equal volume of 0.6 N perchloric acid, to hydrolyse dRib-1-P, (Friedkin & Kalckar, 1950) and dRib-5-P precipitated by the addition of 0.1 ml of $ZnSO_4$ (0.3M) followed by neutralisation with 0.2 ml $Ba(OH)_2$ (0.15 M) and NaOH. dRib-1-P was measured in the supernatant, after centrifugation, and dRib-5-P was released from the precipitate, by treatment with perchloric acid, and measured in the supernatant after further centrifugation. Deoxyribose was measured by the method of Burton (1956). (2) by measuring the formation of the hydrazone of dRib-5-P with phenylhydrazine. This is described in detail later.

Transketolase-Transaldolase activity was measured by coupling the reactions to the reduction of NADP in the presence of excess glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase. Reduction of NADP was dependent on the addition of Rib-5-P which was added to start the reaction. The reaction mixtures contained 2 µmoles of NADP, 10 mµg each of glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase, 0.5 µmoles Rib-5-P and the appropriate amount of extract, in a volume of 1.0 ml made up with

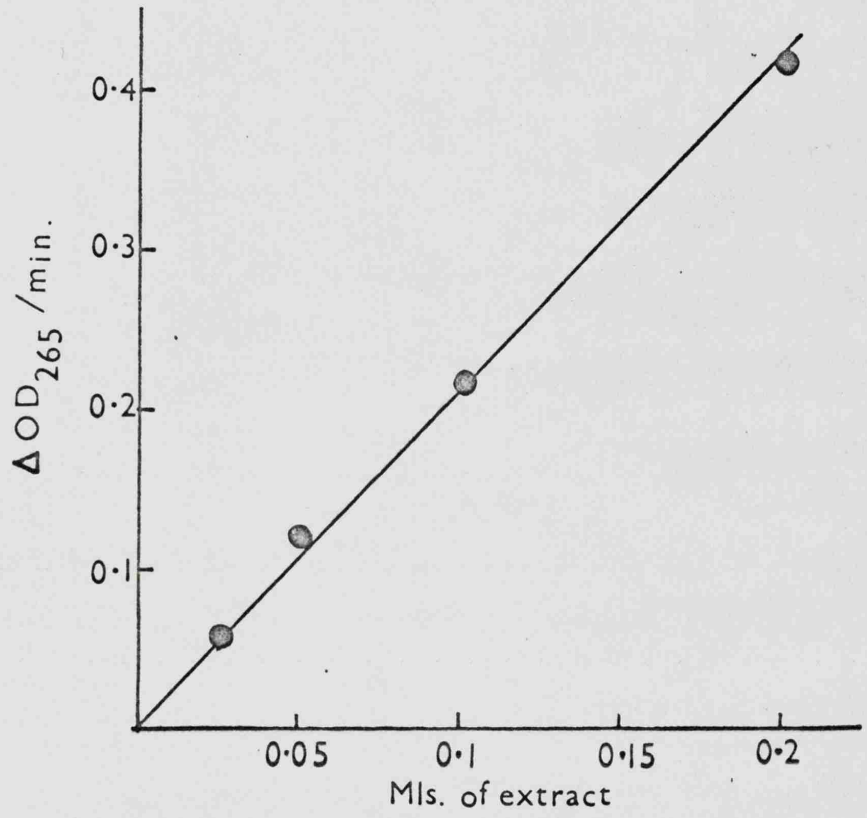
buffer "B". The latter contained 0.9% NaCl (40 ml), 1.15% KCl (1070 ml), 1.75% K_2HPO_4 (200 ml) (pH 5.4), 3.82% $MgSO_4.7H_2O$ (10 ml), adjusted to pH 7.5. The extract (2.0 ml) was incubated with thiamine pyrophosphate (1.8 ml; 100 µg/ml) for 15-30 min before use. It must be emphasised that this assay measured only transketolase-transaldolase activity rather than the complete cycle.

5'-Nucleotidase was assayed in osmotic shock extracts (Neu, 1967a,b) by coupling the reaction to adenosine deaminase (see also Ipata, 1968). The reaction mixture contained 0.25 ml adenosine deaminase, 50 mM $CoCl_2$, 200 mM $CaCl_2$, 0.1 M sodium acetate buffer (pH 6.0), 1 mM 5'-AMP and the appropriate amount of extract in a total volume of 1.0 ml. Proportionality of initial velocity with enzyme concentration is shown in Fig.2. A change in molar extinction coefficient, for the deamination of adenosine, of 8.5×10^3 was used.

Cytidine deaminase was determined by measuring the change in absorbance between deoxycytidine and deoxyuridine at 235 mµ (Peterson et al, 1967). (It was assumed that there was no differences in the absorption of deoxyribo- and ribonucleosides). The reaction mixture contained 50 mM Tris buffer (pH 7.5), 0.1 mM CdR, and the appropriate amount of extract in 1.0 ml. A change in

Fig. 2. 5'-Nucleotidase: Proportionality of initial velocity with Enzyme Concentration

Enzyme was assayed in an osmotic shock extract of E.coli W945-10R which contained 0.23 mg/ml of protein.


0·4
0·3
0·2
0·1
ΔOD265 /min.
0·05
0·1
0·15
0·2
Mls. of extract

molar extinction coefficient of 5.31×10^3 was used.

Uridine phosphorylase was assayed in a similar way to thymidine phosphorylase. The formation of uracil from uridine was followed by measuring the increase in absorbance at 290 mμ at pH 13. A change in molar extinction coefficient of 5.41×10^3 was used (Razzell & Khorana, 1958).

Alkaline phosphatase was assayed according to Garen and Levinthal (1960).

β-galactosidase was measured according to Pardee et al (1959).

Definition of units. One unit of enzyme is defined as the amount which catalyses the conversion of 1 mμmole of substrate per min, or the production of 1 mμmole of product per min.

All assays were carried out at 37°C and for all continuous assays, the reaction was followed using an SP800 recording spectrophotometer.

Deoxyribose was measured according to Burton (1956).

Ribose was measured by the orcinol method (Albaum & Umbreit, 1947).

Changes of media were accomplished by rapid filtration at 37°C using bacterial filters (0.45 μ) (Millipore or Sartorius-Membranfilter GmbH).

Protein was measured by the method of Lowry et al (1951).

Use of activated charcoal. Activated charcoal (obtained from BDH) was acid washed (see Smith & Khorana, 1963) and 1 mg used per ml

of sample. The sample containing activated charcoal was shaken for a few minutes, the charcoal removed by Millipore filtration, and the filtrate analysed. 0.5 µmole of AdR was completely removed from solution by this method, but only about 10% of an equivalent amount of dRib.

Respiration was measured using an oxygen electrode. Exponential phase cells were washed and resuspended in M9 minimal salts medium supplemented with 20 µM thymine and shaken for two hours to deplete endogenous glucose. 0.5 ml of cells (approx. 10^9/ml), and 0.1 ml of TdR (100 mM) where indicated, were made up to a volume of 2.4 ml with 0.1 M phosphate buffer (pH 6.5).).1 ml of glucose (2%) was finally added. The temperature was 30°C.

P1 transduction was performed according to Ahmad and Pritchard (1969).

Mutagenesis with nitrosoguanidine was performed by the method of Adelberg et al (1965).

Graphing of results. In cases where incorporation into, or excretion from, an exponentially growing culture is measured, the function $(2^{t/g}-1)$ is used (where t = time and g = mean generation time). It has been shown that in such a plot the slope of the line is proportional to the rate of incorporation (or excretion) (Barth, 1968; Swenson & Setlow, 1966).

CHAPTER I

A New Assay for Phosphodeoxyribomutase

Until recently assays for phosphodeoxyribomutase were based on chemical differences between dRib-1-P and dRib-5-P and were all sampling assays (see Methods; Munch-Petersen, 1968a; Breitman & Bradford, 1968). Recently the enzyme has been assayed by coupling to deoxyriboaldolase, and measuring the oxidation of NADH (Hoffee & Robertson, 1969). This has the disadvantage, however, of interference due to NADH oxidase. Thus in sonic extracts, the oxidation of NADH by NADH oxidase was always greater than that due to phosphodeoxyribomutase. I have therefore developed the following sensitive, continuous assay for phosphodeoxyribomutase which does not involve the inclusion of any other enzymes in the reaction mixture.

The assay is based on the formation of a hydrazone of dRib-5-P with phenylhydrazine, a principle used in assays involving ketoacids (Dixon & Kornberg, 1959). When dRib-5-P is mixed with phenylhydrazine a compound is formed with a characteristic absorption spectrum which has a peak at 269 mμ (Fig. 3). However, 300 mμ was chosen for the assay due to the high absorption of phenylhydrazine itself, and the bacterial extract, at lower wavelengths. The absorption at 300 mμ is proportional to the quantity

Fig. 3. Absorption Spectrum of the Hydrazone of dRib-5-P

dRib-5-P (0.05 µmole) and phenylhydrazine (0.5 µmole) were mixed in a total volume of 1.0 ml of 0.1 M cacodylate buffer (pH 6.8) and the absorption spectrum recorded in an SP800 spectrophotometer. The reference cell was identical but dRib-5-P was omitted.

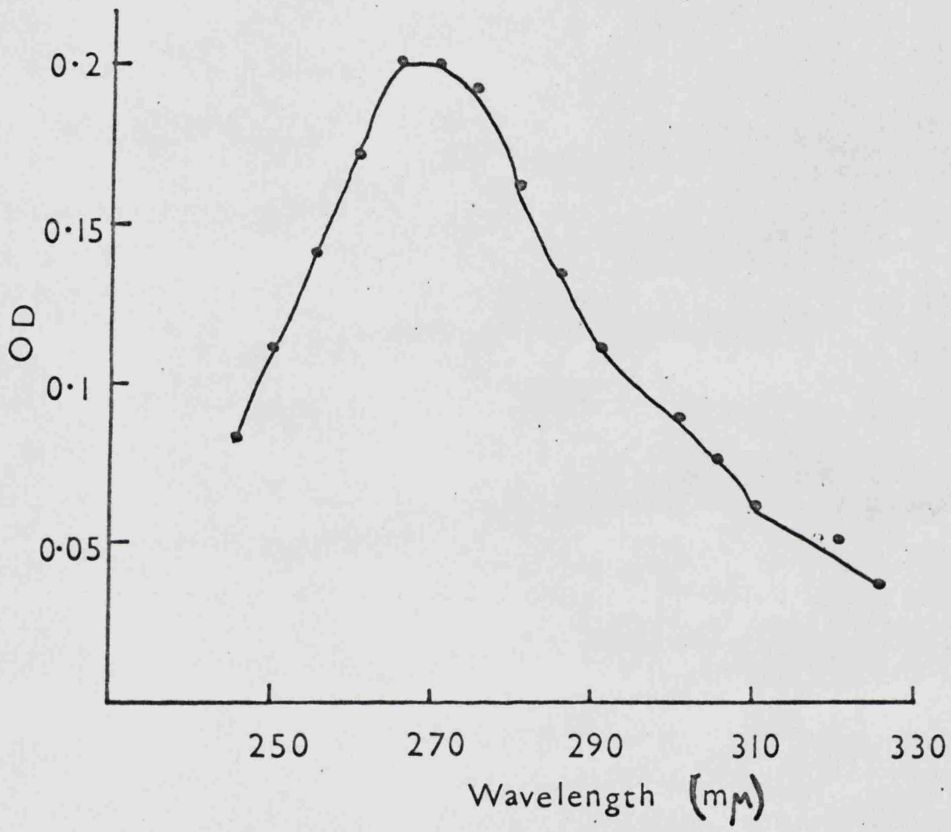
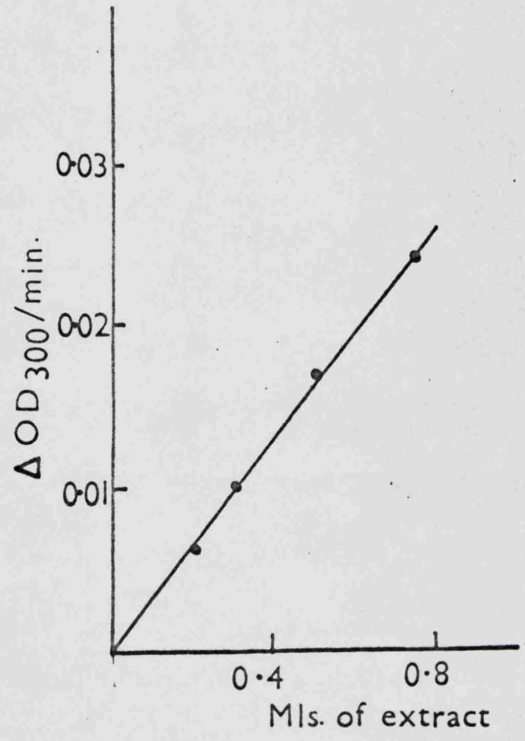

0·2
0·15
0·1
0·05
OD
250
270
290
310
330
Wavelength (mμ)

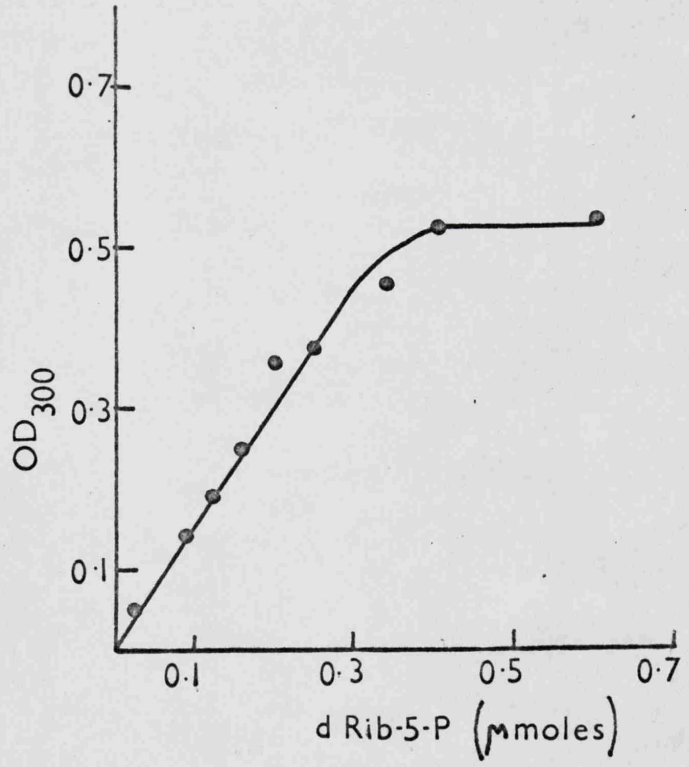
Fig.4. Proportionality of rate of reaction and enzyme concentration

Cells of E.coli CR34 were grown in supplemented glucose minimal medium containing 30 µM thymine, harvested in exponential phase, washed and resuspended in 0.1 M cacodylate buffer (pH 6.8). A sonic extract was then prepared, which contained 1.5 mg/ml of protein, and assayed as described. The reaction was followed for at least 6 min at 37°C in an SP800 spectrophotometer.

Fig.5. Reaction of dRib-5-P with Phenylhydrazine

dRib-5-P and phenylhydrazine (0.5 µmole) were mixed in a total of 1.0 ml of 0.1 M cacodylate buffer (pH 6.8) and the absorbance at 300 mµ (due to dRib-5-P hydrazone) measured after 2-3 min against phenylhydrazine (0.5 µmole). From this curve a molar extinction coefficient of 1.69×10^3 was determined.


0·03
0·02
0·01
ΔOD 300/min.
0·4
0·8
Mls. of extract


0·7
0·5
0·3
0·1
OD300
0·1
0·3
0·5
0·7
d Rib-5-P (μmoles)

of dRib-5-P used (Fig.5). dRib-1-P and Rib-1-P were found not to react with phenylhydrazine since the aldehyde group is esterified. Ribose-5-phosphate (Rib-5-P) also did not react under the assay conditions. In strains of E.coli containing deoxyriboaldolase dRib-5-P is cleaved to give two other aldehydes, acetaldehyde and glyceraldehyde-3-phosphate (Racker, 1952), which also form hydrazones with phenylhydrazine, and could possibly be formed under the conditions of the assay. However, when extracts from a deoxyriboaldolase negative strain were used identical results are obtained whether or not deoxyriboaldolase is added to the reaction mixture. (Osmotic shock extracts from a phospho-deoxyribomutase negative (drm$^-$) strain of S.typhimurium induced with deoxyribose were used as a source of deoxyriboaldolase.)

This indicates that even in the presence of deoxyriboaldolase the dRib-5-P is trapped to form the hydrazone, and acetaldehyde and glyceraldehyde-3-phosphate are not formed. This is presumably due to the large excess of phenylhydrazine present.

The assay mixture contains 0.5 µmole phenylhydrazine, 2.0 µmoles of dRib-1-P, 75 µmoles of buffer such as cacodylate (pH 6.8) and 1-2 mg of bacterial protein in a total volume of 1.0 ml. Assays were performed at 37°C. It was later found that the inclusion of

25 mM sodium fluoride to the cell prior to sonication, resulted in higher activities. The use of phosphate buffer was avoided since phosphate inhibits the enzyme (Hoffman & Lampen, 1952). pH 6.8 was chosen, although the reported pH optimum for the enzyme is 8.5 (Kammen, 1967b; Hoffee & Robertson, 1969), since the rate of the non-enzymic formation of the hydrazone of dRib-5-P rapidly decreases as the pH is raised. Figure 4 shows proportionality of initial velocity with enzyme concentration. It can be seen from Table 4 (see Chapter II) that strains found to be drm$^-$, using a chemical assay (see Methods), also exhibit no phosphodeoxyribomutase with the new assay.

It has been found that the other enzymes of deoxynucleoside catabolism are released by the osmotic shock procedure of Neu and Heppel (1964) (Munch-Petersen, 1967; 1968b; Kammen, 1967a), indicating that they are localised near the cell surface (Neu & Heppel, 1964; Brockman & Heppel, 1968). It was therefore of interest to determine whether this was also true of phosphodeoxyribomutase. The results in Table 2 show that mutase is also released by osmotic shock (see also Munch-Petersen, 1968b) and is therefore also localised near the cell surface.

Munch-Petersen (1968b) observed that whole cells of E.coli have a greater capacity to catabolise the deoxyribose moiety of

TABLE 2 RELEASE OF PHOSPHODEOXYRIBOMUTASE BY OSMOTIC SHOCK

Fraction	Phosphodeoxyribomutase		Thymidine phosphorylase		β-galactosidase	
	Units/ml	% total	Units/ml	% total	Units/ml	% total
1. Sucrose-Tris-EDTA	1.89	5.1	81.4	5.3	5.6	0.2
2. Cold water	27.6	74.4	1144.0	73.8	17.4	0.7
3. Cell pellet	7.6	20.4	325.0	20.9	2400.0	99.1
4. Sonicate	41.0	-	1372.0	-	-	-

Exponential cells of CR34 were washed, resuspended in 5 ml of cacodylate buffer pH 6.8 (0.1 M) at a concentration of 10^{10}/ml and split into two portions of 2.5 ml. One was sonicated to give fraction 4, and the other was subjected to osmotic shock to give fractions 1-3. Osmotic shock involved treatment of the cells with 20% sucrose - 0.03 M Tris (pH 8.0) - 1 mM EDTA followed by rapid dispersion in cold distilled water. The supernatants from both these treatments were retained for assay. The cell pellet left after the cold water treatment was resuspended in 2.5 ml of cacodylate buffer pH 6.8 (0.1 M) and a sonic extract prepared. Thymidine phosphorylase and β-galactosidase assays (see Methods) were used as controls, the former being a known surface enzyme (Kammen, 1967a; Munch-Petersen, 1968a) and the latter an intracellular enzyme (Heppel, 1967).

thymidine than sonic extracts, and it was suggested that mutase was sensitive to sonic disruption of the cells. It can be seen from Table 2, however, that the yield of enzyme released by osmotic shock is similar to that obtained by sonication . Futhermore, the specific activity of mutase when the cells are mechanically disrupted is almost identical to that obtained in sonic extracts. Thus it appears that mutase itself is not sensitive to the sonication procedure used here.

CHAPTER II

Properties of tlr Mutants

(1) Enzymatic Defects and Classification

Considering the known pathways of deoxynucleoside catabolism, as depicted in Fig.1, and the observation by Breitman and Bradford (1967) that one tlr mutant lacks deoxyriboaldolase, it was predicted that tlr mutants should fall into two classes; those lacking deoxyriboaldolase (dra$^-$) and those lacking phosphodeoxyribomutase (drm$^-$). The former should be unable to utilise deoxyribose, whereas the latter should retain this ability. It was indeed observed that tlr mutants of S.typhimurium fell into these two classes (see Table 4). It is possible to show that both classes of mutant are defective in the catabolism of deoxyribose moiety of thymidine by incubating cell-free extracts with thymidine and measuring the total loss of deoxyribose (Table 3). However, this type of experiment is not sufficient to demonstrate a difference between the two classes of tlr mutants. However, the hypothesis is substantiated by directly assaying mutase and aldolase in a sample of tlr mutants (Table 4); those able to utilise deoxyribose are drm$^-$, and some which were unable to do so were dra$^-$. Other laboratories have also noted the absence of mutase and aldolase in tlr mutants (Munch-Petersen, 1968a;

TABLE 3. CATABOLISM OF THYMIDINE IN SONIC EXTRACTS OF DIFFERENT STRAINS OF SALMONELLA TYPHIMURIUM

Strain	Total thymine	Total dRib	dRib-1-P plus free dRib	Free dRib	dRib-1-P	dRib-5-P	dRib disappearance
2006	3.86	1.2	1.27	0.47	0.80	0	2.66
2006-1	1.78	1.5	1.19	0.266	0.924	0.31	0.28
2006-6	1.22	1.1	0.98	0.308	0.672	0.12	0.12

Cultures were induced for one generation of growth with 1 mM thymidine, (in the case of strains 2006 and 2006-6) or with 500 μM thymidine (in the case of 2006-1). The reaction mixture contained 5 μmoles of thymidine, 2 μmoles of phosphate buffer (pH 6.7) and 0.9 ml of sonic extract (approximately 1.0 mg protein in 0.01 M Tris buffer pH 7.5). Samples (0.05 ml) were taken after 3.0 hr into (1) 0.1N NaOH (0.45 ml) for the spectrophotometric determination of thymine at 300 mμ; (2) 0.5 N perchloric acid (0.45 ml) for the determination of total deoxyribose; (3) 0.6 N perchloric acid (0.05 ml). 0.2 M NaOH (0.8 ml) was then added, followed by 0.1 ml of $ZnSO_4$ (0.3 M) and 0.1 ml of $Ba(OH)_2$ (0.15 M). The precipitate was removed by centrifugation and deoxyribose determined in the supernatant. By this treatment dRib-5-P is removed from solution, but not dRib-1-P since it is acid labile and gives free dRib. Thus an estimate is made of the amount of dRib-1-P and free dRib. (4) A sample is taken into chilled distilled water and treated with $ZnSO_4$ and $Ba(OH)_2$ as above. Deoxyribose is determined in the supernatant. This gives an estimate of free dRib, since both dRib-1-P and dRib-5-P are removed from solution by this treatment. In the table, dRib-1-P is given as (column 4 - column 5), dRib-5-P as (column 3 - column 4) and dRib disappearance as (column 2 - column 3). Strains 2006-1 and 2006-6 were derived from 2006 by selection on 20 μM thymine.

TABLE 4. ENZYMATIC DEFECTS AND PROPERTIES OF *tlr* MUTANTS OF *E.COLI* AND *S.TYPHIMURIUM*

Strain	Genotype	Phosphodeoxy-ribomutase	Deoxyribo-aldolase	Growth on dRib[c]	Fermentation of dRib[c]	Sensitivity to	
						TdR	dRib[c]
2006	*thy*$^-$ *drm*$^+$ *dra*$^+$	6.7[a]	78 (430)	+	+	-	-
2006-1	*thy*$^-$ *dra*$^-$	23	0	-	-	+	+
2006-2	*thy*$^-$ *dra*^{c}	29	48.5	-	-	+	+
2006-3	*thy*$^-$ *drm*$^-$	0 (0)[a]	0.75	+	+	-	-
2006-5	*thy*$^-$ *dra*^{c}	31	80	-	-	+	+
2006-6	*thy*$^-$ *drm*$^-$	0	(149)	-	+	-	-
2006-7	*thy*$^-$ *drm*$^-$	0	38	+	+	-	-
2006-9	*thy*$^-$ *dra*^{c}		62	-	-	+	+
2006-10	*thy*$^-$ *dra*$^-$		0	-	-	+	+
2006-12	*thy*$^-$ *dra*^{c}		38.7	-	-	+	+
P152	*thy*$^-$ *drm*$^-$	0 (0)[a]	16[b]	-	-	-	-
CR34	*thy*$^-$ *dra*$^-$	33	0	-	-	+	-

162-8	*thy*⁻ *drm*⁻ *dra*⁻	0	0	-	-	-	-
W945-k9	*thy*⁻ *dra*⁻	14	0	-	-	+	-

All values are given as units/mg of protein.

[a] Assayed by measuring loss of dRib-1-P (see Methods). Other mutase assays were by method (2).

[b] Measured in a sonic extract. Other aldolase assays were by method (2) (see Methods.) Method (2) for the assay of deoxyriboaldolase involves the oxidation of NADH. For this reason osmotic shock extracts were routinely used since the background oxidation of NADH is much reduced in such extracts, as compared to sonic extracts.

( ) Assayed by method (1), using cultures induced with dRib.

[c] Growth on dRib was determined on M9 agar plates containing dRib (10 mM) as sole carbon source and supplemented with thymine. Sensitivity to dRib was determined on plates containing glycerol (25 mM) as carbon source, dRib (10 mM) and thymine. Sensitivity to TdR was determined on plates containing glucose or glycerol as carbon source and thymidine (1mM). Growth was scored after overnight incubation (+ = sensitive; - = not sensitive).

Fermentation of dRib was determined on EMB-nutrient agar plates containing 1% deoxyribose and thymine.

All the derivatives of strain 2006 were obtained by selection on plates containing 20 μM thymine.

Hoffee, 1968; Breitman & Bradford, 1967; 1968). It must be noted, however, that some tlr mutants of S.typhimurium, although they behave nutritionally as dra$^-$ mutants, nevertheless contain deoxyriboaldolase (Table 4; see also Hoffee, 1968). These are tentatively termed dra^c (deoxyriboaldolase, cryptic) mutants. There are thus at least three classes of tlr mutants in S.typhimurium: drm$^-$, dra$^-$ and dra^c. This latter class will be discussed later.

A further correlation was noted between thymidine sensitivity and inability to utilise deoxyribose. Those able to utilise deoxyribose (drm$^-$) were not sensitive, when tested on agar plates (Table 4). Thus it seems that thymidine sensitivity is due to the absence of deoxyriboaldolase. Apart from the significance of this with respect to the mechanism of thymidine sensitivity, it provides a useful means for a preliminary classification of tlr mutants of E.coli K12 which, unlike S.typhimurium, is unable to utilise deoxyribose (Barth et al, 1968). It has so far been found that thymidine sensitive tlr mutants of E.coli are dra$^-$, and those that are not sensitive are drm$^-$ (see Table 4). Dra^c mutants have not yet been found in E.coli.

(2) The Inducer of Enzymes of Deoxynucleoside Catabolism

It was observed by Rachmeler et al (1961) that the phosphorolysis of thymidine in crude extracts was induced by growth of the cultures in medium supplemented with 2 mM thymidine. It was also

noted that the phosphorolysis of purine deoxynucleosides was similarly induced, but to a lesser degree. The induction of thymidine phosphorylase was confirmed by Razzell and Casshyap (1964) who also showed that both purine and pyrimidine deoxynucleosides acted as inducers. The latter observation led these authors to suggest that deoxyribose-1-phosphate might be the actual inducer. More recently, Breitman and Bradford (1967a) suggested that dRib-5-P may be the inducer.

The ease of selection of *drm*$^-$ and *dra*$^-$ mutants presented the opportunity of distinguishing between dRib-1-P and dRib-5-P as the inducer; the use of these strains would not, however, distinguish between dRib-1-P and a deoxynucleoside. However, the data in Table 5 clearly indicate that dRib-5-P is the inducer (see Fig.1). Thus dRib induces both *dra*$^-$ and *drm*$^-$ strains. *Dra*$^-$ strains appear constitutive for thymidine phosphorylase and *drm*$^-$ strains have low induced levels.

It may be noted here that the *dra*^{c} mutants, described above, are also constitutive for thymidine phosphorylase (Table 6).

The induction of the *drm*$^-$ *Salmonella* strains by TdR (Table 5) is assumed to be due to the operation of a by-pass shown in Fig.1. by a dotted line; it is known that *drm*$^-$ strains excrete free dRib when growing on low thymine concentrations and that *tlr* mutants will

TABLE 5. INDUCED AND UNINDUCED LEVELS OF THYMIDINE PHOSPHORYLASE IN *tlr* MUTANTS OF *S.TYPHIMURIUM*

Strain	Genotype	Inducer		
		Nil	Thymidine (2 mM)	Deoxyribose (2 mM)
2006	*thy*$^-$ *tlr*$^+$	54	425	300
2006-1	*thy*$^-$ *dra*$^-$	658	1200	1550
2006-6	*thy*$^-$ *drm*$^-$	47	280	305

Cultures were induced for two generations and enzyme assayed in sonic extracts. Activities are expressed in units/mg protein.

TABLE 6. THYMIDINE PHOSPHORYLASE IN *tlr* MUTANTS OF *S.TYPHIMURIUM*

Strain	Genotype	Specific activity (Units/mg protein)
2006	*thy*$^-$ *thr*$^+$	109
2006-1	*thy*$^-$ *dra*$^-$	1425
2006-2	*thy*$^-$ *dra*^{c}	2262
2006-3	*thy*$^-$ *drm*$^-$	80
2006-6	*thy*$^-$ *drm*$^-$	157
2006-7	*thy*$^-$ *drm*$^-$	34
2006-9	*thy*$^-$ *dra*^{c}	3170
2006-10	*thy*$^-$ *dra*$^-$	2528

Cultures were grown on low thymine (20 μM), and harvested in exponential growth.

excrete the dRib moiety of thymidine added to the medium (Barth, 1968). Presumably, in Salmonella typhimurium, this dRib can then be phosphorylated via deoxyribokinase (Hoffee, 1968).

The hypothesis that dRib-5-P is the inducer of thymidine phosphorylase predicts that in E.coli K12, which cannot utilise dRib, no induction at all should be observed in drm$^-$ strains and this has been confirmed (Table 7; Barth et al, 1968). The results in Table 7 show that not only thymidine phosphorylase, but also other enzymes of deoxynucleoside catabolism are induced by dRib-5-P.

Several recent reports seem to confirm the conclusion that dRib-5-P is the inducer of thymidine phosphorylase. Hoffee (1968) has reported that some dra$^-$ strains are constitutive for thymidine phosphorylase. Munch-Petersen (1967) has also reported that some tlr mutants have high thymidine phosphorylase levels, one of which (E.coli B_3) has since been shown to be dra$^-$ (Munch-Petersen, 1968a). Razzel and Casshyap (1964) reported that some thy$^-$ mutants are uninducible for thymidine phosphorylase: it seems probable that these are drm$^-$. Breitman and Bradford (1968) have shown that several drm$^-$ strains are non-inducible, and also conclude that dRib-5-P is the inducer of thymidine phosphorylase and deoxyribo-aldolase.

TABLE 7. INDUCTION BY THYMIDINE OF ENZYMES OF DEOXYNUCLEOSIDE CATABOLISM IN E.COLI

Strain	Genotype	Purine phosphorylase		Thymidine phosphorylase		Deoxyribo-aldolase		Phosphodeoxy-ribomutase	
		+	-	+	-	+	-	+	-
C600	thy$^+$ tlr$^+$	620	123	1356	97	893	164		3.0
P152	thy$^-$ drm$^-$	101	105	115	138	57	49		0
CR34	thy$^-$ dra$^-$		850		2710		0		33

All values are given as units/mg protein.

Induction was for one generation of growth with 2 mM thymidine.

+ = induced; - = non-induced.

It might be noted that the endogenous induction of enzymes of deoxynucleoside catabolism by dRib-5-P in thy⁻ dra⁻ strains is not a unique case. A similar phenomenon occurs in the gal operon (Wu & Kalckar, 1966) and in the histidine degradative pathway of Aerobacter aerogenes (Schlesinger et al, 1965).

With the identification of dRib-5-P as the inducer, an opportunity exists to determine whether endogenous induction plays any role in the determination of the basal (uninduced)level of synthesis of thymidine phosphorylase. The basal level of thymidine phosphorylase was determined in three isogenic strains of E.coli K12 (Table 8). However, no significant difference was observed between the wild-type and a drm⁻ strain indicating that any endogenous dRib-5-P derived from deoxynucleosides does not play a role in regulating the basal level of synthesis. This confirms results obtained with E.coli C600 and P152 (Table 7). (Although these latter strains can be traced to a common parent, they are not necessarily isogenic, having been subjected to different selective pressures during the past: they are thus not strictly comparable). Similar conclusions were made by Wu and Kalckar (1966) in the case of the galactose system, using analogous data. It has also been shown that the basal level of β-galactosidase in E.coli K12 and E.coli 15 strains is not reduced by a competitive inhibition of

TABLE 8. UNINDUCED LEVELS OF THYMIDINE PHOSPHORYLASE IN ISOGENIC *tlr* MUTANTS OF *E.COLI*

Strain	Genotype	Thymidine phosphorylase (units/mg)
W945-10	*thy*$^-$ *dra*$^-$	1860
W945-15	*thy*$^-$ *drm*$^-$	76
W945-3	*thy*$^-$ *tlr*$^+$	59

induction which is believed to bind to the repressor and thereby prevent inducer-repressor interaction (Yayaraman et al, 1966; Overath, 1968). This is taken as evidence for the absence of endogenous induction being responsible for the basal level of β-galactosidase.

(3) The Possible Induction of Other Enzymes by dRib-5-P

Two facts strongly indicate that the genes specifying phosphodeoxyribomutase, deoxyriboaldolase, thymidine phosphorylase and purine phosphorylase, constitute an operon. Firstly, all four enzymes cluster together on the E.coli map (Alikhanian et al, 1966; Ahmad et al, 1968) and the gene order is now known (Ahmad & Pritchard, 1969). Secondly, all four enzymes have a common inducer in dRib-5-P.

It seemed worthwhile, therefore, to investigate the possibility that other enzymes responsible for the catabolism of deoxynucleosides and deoxynucleotides might also be inducible by thymidine and map close to the tlr locus. Two such enzymes are cytidine deaminase (Cohen & Barner, 1957; Karlstrom, 1968), and 5'-nucleotidase (Neu, 1967a,b). The latter is a surface located enzyme and has an intracellular inhibitor. It can therefore only be assayed in osmotic shock extracts (see Methods) or heat-treated sonic extracts (Neu, 1967a,b; 1968). Both enzymes are specific for the ribo- and deoxyribo- compounds. 5'-nucleotidase was considered in view of the

fact that the source of deoxyribonucleosides is unknown, but are currently assumed to arise from deoxyribonucleotides (see Chapter III).

The data in Tables 9 and 10 show that neither cytidine deaminase nor 5'-nucleotidase are induced by TdR in strain C600 and are not constitutive in CR34.

Strains lacking cytidine deaminase (*cdd*$^-$) were obtained in W945-10R by isolating fluorodeoxycytidine resistant mutants (Karlstrom, 1968). The *tpp*, *dra*, *pup* and *drm* loci are all about 95% co-transducible with *ser*B. To determine if this was also the case with the *cdd* locus, W945-10R *cdd*$^-$ was used as donor and strain 537 (*ser*B$^-$) as recipient in a transductional cross using phage P1. *Ser*$^+$ transductants were selected and about twenty were tested for resistance to FCdR, but none were resistant. It is concluded that *cdd* is unlinked to *ser*B.

It was attempted to isolate strains lacking 5'-nucleotidase by isolating mutants unable to grow on 5'-adenylic acid (AMP) as sole carbon source, but still able to grow on deoxyadenosine. The cells were previously mutagenised with nitrosguanidine. About ten mutants with this phenotype were isolated from W945-10R, but they, very surprisingly, possessed normal levels of 5'-nucleotidase when the enzyme was assayed in osmotic shock extracts. A similar mutant, isolated by a different method, was simultaneously reported by

TABLE 9. INDUCTION OF ENZYMES OF DEOXYNUCLEOSIDE CATABOLISM BY VARIOUS NUCLEOSIDES

Strain	Inducer	Thymidine phosphorylase	Purine phosphorylase	Cytidine deaminase
(1) C600	Nil	161	122	74
	cytidine	98	105	143
	uridine	65	102	75
	thymidine	961	259	78
(2) CR34	Nil	2118		156
C600	Nil	128		120

Cultures were induced for one generation of growth with 2 mM nucleoside, and enzymes assayed in sonic extracts. All values are expressed as units/mg protein.

TABLE 10. NON-INDUCTION OF 5'-NUCLEOTIDASE BY THYMIDINE

Strain	Genotype	Presence of TdR (2 mM)	5'-Nucleotidase	Thymidine phosphorylase
C600	*thy*$^+$ *tlr*$^+$	+	1318	1550
C600	*thy*$^+$ *tlr*$^+$	-	1070	205
CR34	*thy*$^-$ *dra*$^-$	-	1325	2862

Cultures were induced with thymidine (2 mM) for one generation of growth. 5'-nucleotidase was assayed in osmotic shock extracts and phosphorylase was assayed in sonic extracts. Values are in units/mg protein.

Ward and Glaser (1968). Osmotic shock extracts of their mutant had full activity, but whole cells were unable to hydrolyse 5'-AMP. On the basis of this, and other experiments, they suggest that their mutant is cryptic; more specifically, that the surface orientation of the enzyme is altered such that it is unable to hydrolyse an external substrate. It seems very likely that the mutants unable to grow on 5'-AMP are similar.

CHAPTER III

The Source of Deoxyribose in tlr Mutants

(1) The Role of the thy Locus: An Hypothesis for the Regulation of the Production of Deoxyribose-Phosphates.

The absence of phosphodeoxyribomutase or deoxyriboaldolase in tlr mutants strongly suggests that the low thymine requirement of these strains is due to an expanded dRib-1-P pool, allowing an efficient incorporation of thymine via thymidine phosphorylase. (It will be shown later that incorporation of thymine does in fact normally occur exclusively via thymidine phosphorylase). The constitutivity of thymidine phosphorylase in thy$^-$ dra$^-$ strains is presumably a result of an expanded dRib-5-P pool. However, as mentioned earlier, wild-type strains of E.coli are totally unable to incorporate external thymine, indicating that they have a negligible dRib-1-P pool. We therefore considered that the formation of dRib-1-P and dRib-5-P in thy$^-$ mutants (and its accumulation in thy$^-$ tlr$^-$ mutants) may be a consequence of the thy$^-$ mutation rather than the tlr mutation. In order to examine this possibility, thy$^+$ derivatives of some thy$^-$ dra$^-$ mutants were made and the levels of thymidine phosphorylase measured. The data in Table 11 show that the constitutivity of thymidine phosphorylase observed in dra$^-$ strains is only apparent in strains which are

TABLE 11. SPECIFIC ACTIVITY OF THYMIDINE PHOSPHORYLASE IN DIFFERENT STRAINS OF E.COLI AND S.TYPHIMURIUM

Strain	Genotype		Thymidine phosphorylase (units/mg)
	thy	dra	
C600	+	+	58
W945	+	+	61
W945-3	-	+	59
W945-10	-	-	1650
W945-10R	+	-	129
2006	-	+	87
2454	-	-	1033
2006-1	-	-	1700
2456	+	-	42

Thy^- dra^+ strains were grown in medium supplemented with 200 µM thymine. Thy^- dra^- strains were grown in 20 µM thymine. Cultures were harvested in exponential phase and enzyme determined in sonic extracts.

also thy⁻; strains which have been reverted to thymine independence have basal (wild-type) levels of thymidine phosphorylase. Thus dRib-5-P (and presumably, therefore, dRib-1-P) is only formed in thy⁻ strains. The following hypothesis is proposed to account for this observation. It will be assumed that dRib-1P and dRib-5-P arise via catabolism of deoxynucleotides. This assumption is examined in more detail later.

Consider the consequences of a thy⁻ mutation leading to a thymine requirement. Assume that initially the pool size of dRib-1-P is negligible. There will therefore be no synthesis of thymidylate from external thymine (via thymidine phosphorylase, thymidine kinase, TMP kinase and TDP kinase) and the internal TTP concentration will fall. It is known that TTP plays a key role in regulating the activity of ribonucleoside diphosphate reductase. TTP inhibits the activity, in the presence of ATP, of this enzyme (Larsson & Reichard, 1967; Reichard, 1967; Brown & Reichard, 1969 a,b). Also a thymine derivative, possibly TTP, represses the synthesis of this enzyme (Biswas et al, 1965). Reduction of the intracellular concentration of TTP should therefore lead to an increase in the intracellular concentration of the other deoxynucleoside triphosphates, and this has been shown to occur (Neuhard & Munch-Petersen, 1966; Neuhard, 1966). The consequence of a thy⁻ mutation will therefore be a decrease in the TTP concentration,

an increase in concentration of other deoxynucleotides and, it is postulated, catabolism of these to give dRib-1-P and dRib-5-P. Synthesis of thymidylate will now commence and its rate will depend on (a) the concentration of thymine and (b) the concentration of dRib-1-P. The higher the thymine concentration the greater will be the rate of synthesis of thymidylate and the lower the rate of breakdown of deoxynucleotides to dRib-1-P. The system will thus be self-regulating in the sense that the external thymine concentration will determine the rate of breakdown of deoxynucleotides, which in turn will determine the rate of synthesis of TTP. The size of the TTP pool,at equilibrium, will therefore be dependent on the external thymine concentration. Since in *dra*$^-$ or *drm*$^-$ strains the further catabolism of dRib-phosphates is blocked, a given rate of breakdown of deoxynucleotides will lead to a higher internal concentration of these compounds and consequently permit normal growth on correspondingly lower thymine concentrations. The key observation which has led to this hypothesis is that in *dra*$^-$ strains which have normal thymidylate synthetase activity there is no constitutivity of thymidine phosphorylase or excretion of dRib (Table 8; Beacham *et al*, 1968b). (It must be mentioned here that excretion of dRib also is assumed to be due to the expanded dRib-phosphate pool.) This behaviour indicates that breakdown

of deoxynucleotides to dRib-phosphates is due to the thy⁻ mutation.

One consequence of this scheme is that we should expect that increasing the concentration of thymine in thy⁻ dra⁻ strains should progressively decrease the specific activity of thymidine phosphorylase by lowering the concentration of dRib-phosphates (a) directly by shifting the equilibrium of the thymidine phosphorylase reaction in favour of TdR and (b) indirectly, by raising the internal concentration of TTP and thus reducing the rate of breakdown of deoxynucleosides. This prediction is confirmed by the data in Table 12. The excretion of dRib behaves in the same way (Beacham et al, 1968b). Growth in the presence of 1 mM thymine reduces the specific activity of thymidine phosphorylase by up to ten-fold. The specific activity found when 1 mM thymine concentrations are used is still higher than the basal level found in dra⁻ strains. This is to be expected on our hypothesis, since, if deoxynucleotide breakdown is necessarily involved in thymine incorporation, then there will be a limit to the extent to which the TTP pool size can be raised, by increasing the thymine concentration, without decreasing the rate of production of deoxyribose-phosphates to a level which itself limits the rate of synthesis of TTP from external thymine.

It may be noted here that (a) above cannot be solely responsible for the effect of thymine concentration on constitutivity since

TABLE 12. EFFECT OF THYMINE CONCENTRATION ON THE LEVEL OF THYMIDINE PHOSPHORYLASE IN *thy*⁻ *dra*⁻ STRAINS OF *E.COLI* AND *S.TYPHIMURIUM*

Strain	Genotype	Thymidine phosphorylase (units/mg)		
		Thymine concentration		
		20 µM	200 µM	1 mM
CR34	*thy*⁻ *dra*⁻	2710	570	239
W945-10	*thy*⁻ *dra*⁻	1650	569	270
2006-1	*thy*⁻ *dra*⁻	1700	1116	465

Cultures were grown to 2 x 10^8 cells/ml, harvested and enzyme determined in sonic extracts.

growth of a thymidine phosphorylase negative mutant (tpp$^-$) of CR34 on thymidine has the repressed level of purine phosphorylase (Table 13). In this case there is no possibility of lowering the deoxyribose-phosphate pool by reaction of dRib-1-P with thymine. The interesting feature of the data in Table 13 is that even on very low thymidine concentrations the repressed level of purine phosphorylase is observed. It may be postulated that the rate limiting reaction in the conversion of thymine to dTTP is the conversion of thymine to thymidine via thymidine phosphorylase. Thus, on this hypothesis, the pool size of dTTP is maintained at the normal (wild-type) level, or higher, even on very low thymidine concentrations. That is, the dTTP pool size is indpendent of the external thymidine concentration, and is high.

Another factor may be involved in the dependence of constitutivity on thymine requirement. This derives from the fact that in the thy$^-$ mutants examined, the further catabolism of dUMP via thymidylate synthetase is blocked (see Chart 1; Barner & Cohen, 1959; Mantsavinos & Zamenhof, 1961; Breitman & Bradford, 1967). It is possible therefore that an accumulation of dUMP may result. If dRib-phosphates arise mainly from uridine deoxynucleotides and the latter accumulate in a thy$^-$ mutant, then removing the block, as in a thy$^+$ mutant, might be expected to

TABLE 13. PURINE NUCLEOSIDE PHOSPHORYLASE IN A *tpp*⁻ MUTANT GROWN WITH VARIOUS CONCENTRATIONS OF THYMIDINE

Concentration of TdR in medium (μM)	Purine phosphorylase (units/mg protein)
1	182
2	278
5	226
10	310
20	335
200	255
0	1760

E.coli 162-19 (derived from CR34) requires thymidine as a thymine source. An exponential culture growing in glucose M9 medium supplemented with 0.4% casamino acids and 20 μM thymidine was filtered, washed and resuspended in similar medium containing the concentration of thymidine indicated. The starting cell titre was 10^6/ml and cultures were harvested at 5 x 10^7/ml and purine nucleoside phosphorylase assayed in sonic extracts.

abolish constitutivity of thymidine phosphorylase. However, whether or not dUMP would accumulate depends on what feedback controls exist at this stage in nucleotide metabolism. Bertani *et al* (1963) have found that the *in vivo* reduction of UDP to dUDP is strongly inhibited by dUDP and by dUTP and suggest that this may be part of a homeostatic mechanism *in vivo*. The possibility that dUMP accumulates is thus less likely.

Accumulation of dUMP cannot solely account for the effect of thymine requirement on constitutivity since, as argued above in the case of the effect of thymine concentration, repressed levels of purine phosphorylase are observed in a *thy*$^-$ strain grown on thymidine as a thymidylate source.

It might be emphasised that the two mechanisms suggested above are not mutually exclusive. Thus accumulation of deoxyuridine nucleotides may occur, but the extent of their formation, and accumulation, may depend on the size of the dTTP pool.

The important conclusion that is drawn from the above observations and their interpretation, is that gross catabolism of deoxynucleotides to dRib-phosphates (with concomitant induction of thymidine phosphorylase and excretion of the dRib moiety as the free sugar in *dra*$^-$ mutants) does not normally occur, but is a result of a disturbance in the control of deoxynucleotide synthesis

which results from the thymine requirement of *thy*$^-$ strains.

There are some reports in the literature which seem to partially conflict with the observations described above. Crawford (1958) has noted, using *E.coli* B_3 and *E.coli* 15T$^-$, that reversion to thymine independence results in only a reduced capacity to incorporate exogenous labelled thymine (50% and 25% of the *thy*$^+$ derivative respectively). *E.coli* B_3 is *dra*$^-$ (Munch-Petersen, 1967, 1968a) and *E.coli* 15T$^-$ is *drm*$^-$ (Breitman & Bradford, 1968). If the catabolism of deoxynucleotides to give dRib-phosphates is abolished in a *thy*$^+$ strain, as postulated above, then incorporation of thymine should not occur. Secondly, Munch-Petersen (1967) found that some *thy*$^-$ *tlr*$^-$ strains have high levels of thymidine phosphorylase but that *thy*$^+$ revertants and transductants have a reduced level of enzyme, which is higher than the basal level. (Recently, however, the same author has noted that *dra*$^-$ strains, selected by their inability to grow on deoxynucleosides, when made *thy*$^-$ by the trimethoprim method, become constitutive (Munch-Petersen, personal communication)). Thirdly, Harrison (1965) finds that revertants of *E.coli* 15T$^-$ to *thy*$^+$ fall into two classes: a majority that are indistinguishable from wild type, possessing a low rate of thymine incorporation, and a few which incorporated thymine at the same rate as strain 15T$^-$. It was also found that the revertants of the

latter class grew diphasically unless 1 μg/ml (8 μM) of thymine was present. This characteristic was lost on storage for several months at 5°C. The most significant observation, however, is that the majority class possess wild-type levels of thymidylate synthetase whereas the minority class have a very low specific activity (about 10% of wild type) of this enzyme. Likewise with Aerobacter aerogenes, thy$^+$ revertants of thy$^-$ mutants fell into two classes: a majority which grew normally and had normal levels of thymidylate synthetase, and a minority class which required 1 μg/ml thymine for normal growth and possessed low levels of thymidylate synthetase. Thymine incorporation was not measured. Revertants of another E.coli 15 thy$^-$ strain gave only revertants which were indistinguishable from wild-type showing the low rate of thymine incorporation.

In view of these results it seems likely that all the revertants to thy$^+$ which still behave like tlr mutants have very low thymidylate synthetase activity. That is, they are only partial revertants, and therefore might have a low TTP pool size; they should therefore have a correspondingly high rate of catabolism of deoxynucleotides, allowing a relatively high rate of external thymine incorporation. This explanation does not hold, of course, for the thy$^+$ transductants of Munch-Petersen (1967) described above, unless they are in fact (partial) revertants which appeared among the transductants.

Mantsavinos and Zamenhof (1961) studied the conversion of thymine to thymidine *in vitro*. They showed that a *thy*$^+$ revertant, of what is now known to be a *tlr*$^-$ mutant, had the ability to convert thymine to thymidine in dialysed extracts. This strain was also a partial revertant. The puzzling feature of their data however, is that the wild-type strain (*thy*$^+$ *tlr*$^+$) also possessed this pathway. It is perhaps possible that a breakdown of deoxynucleotides to deoxyribosyl compounds was occurring in their extracts.

The *thy*$^-$ *dra*$^-$ strain used by Breitman and Bradford (1967b; 1968) does not appear to be constitutive for thymidine phosphorylase. This is probably due to two factors: firstly they grew their cultures on 1 mM thymine. As shown above, high concentrations of thymine tend to reduce the degree of constitutivity. Secondly, this strain has very low mutase activity (Breitman & Bradford, 1968) which might not allow a sufficient rise in the dRib-5-P concentration, during growth on thymine, for thymidine phosphorylase to become induced.

Some observations by Barth (1968) are also highly relevant to the hypothesis outlined above. He found that treatment of some *tlr* mutants with nalidixic acid, or with ultraviolet light, brings about an excretion of deoxyribose in a similar way to thymine starvation (Breitman & Bradford, 1964). Nalidixic acid is a drug

which specifically inhibits DNA synthesis (Goss *et al*, 1964), although its exact mode of action is not yet known. Thus all three treatments cause a specific inhibition of DNA synthesis, and excretion of deoxyribose. (As mentioned earlier, the excretion of deoxyribose in *tlr* mutants is assumed to be a consequence of an expanded deoxyribose-phosphate pool.) We postulate that upon inhibition of DNA synthesis, deoxynucleotides accumulate, and are catabolised to give deoxyribose. Support for the former occurring is provided by Smith and Davis (1967) who showed that deoxynucleotides accumulate when DNA synthesis is inhibited by novobiocin. The effect of nalidixic acid occurs in both *thy*$^-$ *tlr*$^-$ and *thy*$^+$ *tlr*$^-$ strains (Barth, 1968) and also on high (1 mM) thymine concentrations (see Table 14). Thus, at first sight, the above explanation of the results of an inhibition of DNA synthesis seems to conflict with the hypothesis that TTP regulates deoxynucleotide synthesis, and hence deoxynucleotide breakdown. However, it is possible that deoxynucleotides accumulate, irrespective of a normal TTP pool level, when their utilisation is blocked. The fact that nalidixic acid induced excretion of deoxyribose occurs in *thy*$^+$ strains, also shows that the accumulation of only deoxyuridine nucleotides is not necessarily involved.

Thus deoxynucleotides may accumulate, and be catabolised, under two conditions: firstly, a block in their utilisation for DNA synthesis, and secondly, if the TTP pool is specifically lowered.

It may be noted at this point that it can be calculated that neither breakdown of the existing deoxynucleotide pools, nor DNA, can quantitatively account for the amount of deoxyribose excreted. Thus breakdown of the total deoxynucleoside triphosphate pool (Neuhard, 1966) would yield the equivalent of 0.07 mμmoles of dRib/10^8 bacteria. Breakdown of 1.4 genome equivalents of DNA would yield 1.82 mμmoles of dRib/10^8 bacteria. The rate of dRib excretion observed during nalidixic acid treatment, UV-irradiation or thymine starvation of a tlr mutant is at least 3 mμmoles/10^8 bacteria/generation (Barth, 1968). Furthermore this rate is maintained for at least three doublings of cell mass and therefore a net synthesis of the source of deoxyribose is required.

(2) The Pathway of Deoxyribose-Phosphate Production

In the previous section it was assumed that TTP regulates ribonucleotide reductase by repression and feedback inhibition, thereby regulating the synthesis of deoxynucleotides. It was proposed that deoxynucleotides were catabolised to give dRib-phosphates and that the extent of this catabolism was regulated

by the size of the TTP pool. The following discussion is concerned with the route by which deoxyribose-phosphates arise in tlr mutants.

As emphasised earlier, no pathway is known by which deoxyribonucleosides may arise in the cell, yet they are the obvious candidates for the immediate precurosr of deoxyribose-phosphates (see Fig.1). Phosphorylases are known which split deoxyribonucleosides to dRib-1-P. Like phosphodeoxyribomutase and deoxriboaldolase, which are concerned with the metabolism of deoxyribose-phosphates, thymidine phosphorylase and purine phosphorylase are induced by dRib-5-P (see Chapter II) and all four enzymes cluster together on the E.coli map (Ahmad & Pritchard, 1969). The simplest hypothesis, therefore, is that deoxynucleotides are catabolised to give deoxynucleosides which are then degraded by known phosphorylases. The synthesis and interrelationships of deoxynucleotides are shown in Chart 1, together with pathways of ribonucleoside and deoxynucleoside metabolism.

It is clear from the previous discussion that any inhibitor of DNA synthesis which acts at the level of deoxynucleotide synthesis should not cause excretion of deoxyribose. Such an inhibitor is hydroxyurea (HU). There is very good evidence that this agent inhibits ribonucleotide reductase (Neuhard, 1967; Krakoff et al, 1968). An attempt was made, therefore, to substantiate the hypothesis

CHART 1. NUCLEOTIDE AND NUCLEOSIDE METABOLISM

In most cases references are given. These are not intended to be comprehensive, nor do they necessarily refer to the original discovery of the enzyme concerned: rather the latest reference or the one considered to be most useful is given.

1. Thymidine phosphorylase (Rachmeler et al, 1961; Razzell & Khorana, 1958)
2. Purine phosphorylase (Rachmeler et al, 1961; Ahmad et al, (1968)
3. Phosphodeoxyribomutase (Manson & Lampen, 1951)
4. Deoxyriboaldolase (Racker, 1952)
5. Deoxyribokinase (Hoffee, 1968)
6. Cytidine deaminase (Cohen & Barner, 1957; Karlstrom, 1968)
7. Uridine phosphorylase (Page & Schlenk, 1952; Krenitsky et al, 1964)
8. Adenosine deaminase (Remy & Love, 1968)
9. See Mantsavinos and Zamenhof (1961); Denhardt (1969)
10. Ribokinase (Anderson & Cooper, 1969)
11. 5'-phosphoribosyl-1-pyrophosphate synthetase (Kornberg et al, 1955)
12. Uridine-5'-monophosphate pyrophosphorylase (Crawford et al, 1957; Molloy & Finch, 1969)

Contd. p. 65

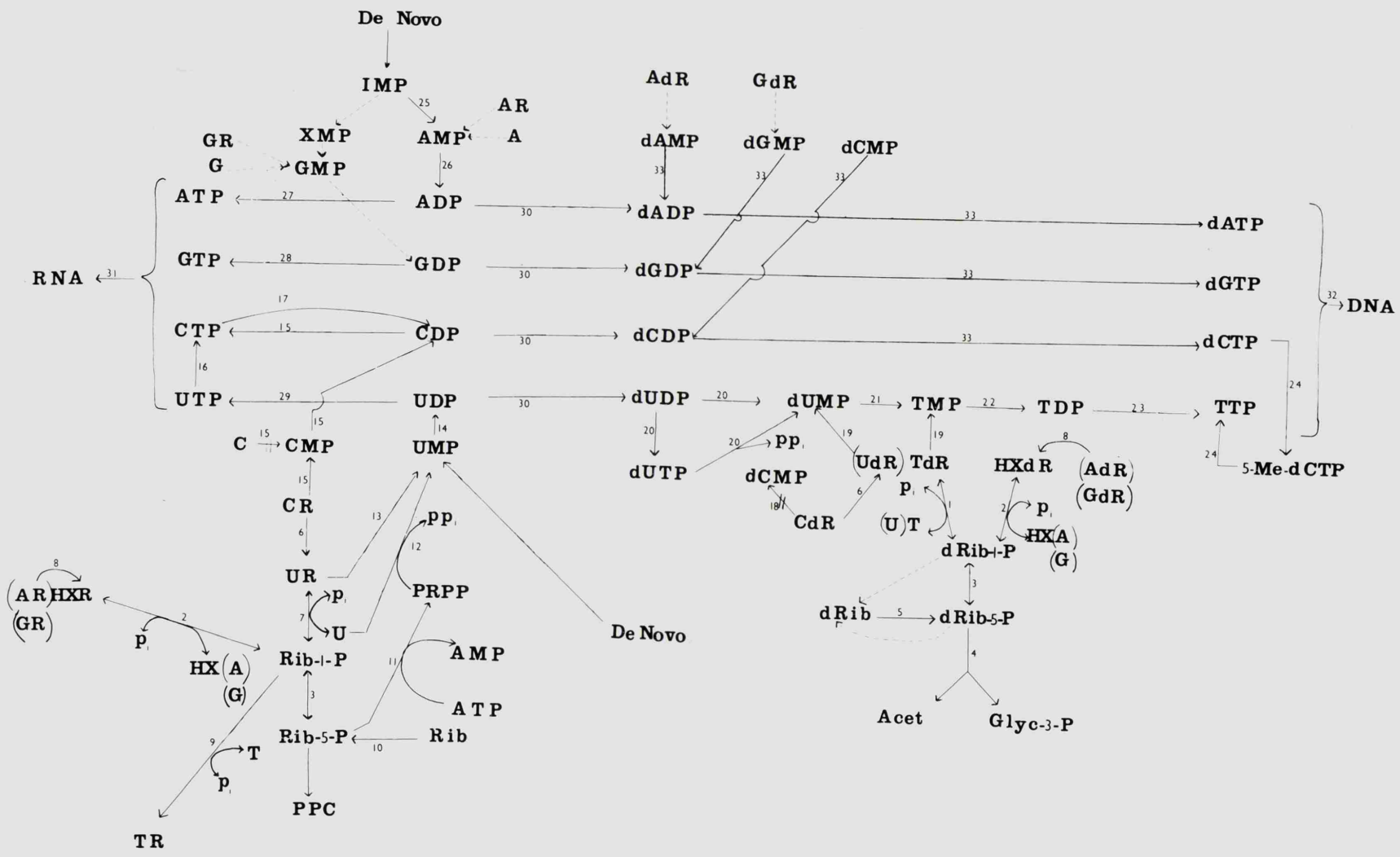

De Novo
IMP
AR
A
AdR
GdR
GR
G
XMP
GMP
AMP
dAMP
dGMP
dCMP
ATP
ADP
dADP
dATP
GTP
GDP
dGDP
dGTP
RNA
DNA
CTP
CDP
dCDP
dCTP
UTP
UDP
dUDP
dUMP
TMP
TDP
TTP
5-Me-dCTP
C
CMP
UMP
dUTP
pp
dCMP
CdR
UdR
TdR
HXdR
AdR
GdR
CR
UR
PRPP
U
HX
A
G
AR
HXR
GR
Rib-1-P
dRib-1-P
dRib
dRib-5-P
AMP
ATP
Rib-5-P
Rib
T
TR
PPC
Acet
Glyc-3-P

CHART 1 (contd.)

13. See Roberts et al (1963)

14. See Bertani et al (1963)

15. See Neuhard & Ingraham (1968)

16. Cytidine triphosphate synthetase (Long & Pardee, 1967)

17. This is presumed to be the normal route for the synthesis of CTP

18. See Neuhard (1968)

19. Thymidine kinase (Okazaki & Kornberg, 1964; Hiraga et al, 1967)

20. See Bertani et al (1963)

21. Thymidylate synthetase (Barner & Cohen, 1959)

22. TMP kinase (Nelson & Carter, 1969)

23. TDP kinase (see Lehman et al, 1958)

24. Forster & Holldorf, (1965)

25. See Moat & Friedman (1960)

26. Adenylate kinase (E.C. 2.7.4.3.)

27. }
28. } Nucleoside diphosphate kinases
29. }

30. Ribonucleotide reductase (see Reichard, 1968)

31. RNA polymerase

32. DNA polymerase

33. Deoxyribonucleotide kinases (see Lehman et al, 1958)

PPC = pentose phosphate cycle. For other abbreviations see p.

that deoxynucleotides give rise to deoxyribose-phosphates by comparing the effects of HU, nalidixic acid, and thymine starvation on tlr mutants. Thus HU should not bring about the formation of dRib-phosphates itself, and should suppress the formation of deoxyribose-phosphates brought about by thymine starvation.

In the following experiments, the production of deoxyribose-phosphates was measured in two ways: (1) the induction of either of the phosphorylases, or phosphodeoxyribomutase when cells are thymine starved. The amount of enzyme is taken to be an approximate measure of the dRib-5-P pool. (2)The excretion of deoxyribose. (It is not known whether free dRib arises from dRib-1-P or both dRib-1-P and dRib-5-P).

The hyper-induction of thymidine phosphorylase and excretion of dRib by nalidixic acid and HU was determined in a dra$^-$ strain (CR34) under various conditions (Table 14). The data certainly indicate that HU and nalidixic acid have different effects. Nalidixic acid, like thymine starvation causes excretion of deoxyribose (Barth, 1968) and induction of thymidine phosphorylase, whereas with HU these effects are greatly reduced. Some excretion of deoxyribose is seen in HU treated cultures; this could be due to incomplete inhibition of the reductase. More specifically, HU might only inhibit the reduction of the particular nucleotides involved in

TABLE 14. THYMIDINE PHOSPHORYLASE AND DEOXYRIBOSE EXCRETION IN E.COLI CR34 UNDER VARIOUS CONDITIONS

Additions to culture[a]			Thymidine phosphorylase (units/mg protein)	Deoxyribose excretion (mμmoles/ml of culture)
Thy	Nal	HU		
+	-	-	296	4.2
+	+	-	4440	52
+	-	+	260	10
-	-	-	5476	46
-	-	+	-	9.3

[a] Thy = presence of 1mM thymine; Nal = 50 μg/ml nalidixic acid; HU = 0.1 M hydroxyurea.

Cultures were grown in glucose-M9 medium supplemented with 0.4% casamino acids and 1 mM thymine. At a cell concentration of 2 x 10^8/ml, cultures were filtered, washed, and resuspended in glucose-M9 medium supplemented with 0.4% casamino acids and the additions indicated in the Table. After 3 hr, samples were taken for the estimation of deoxyribose. The rest of the culture was harvested, and thymidine phosphorylase assayed in sonic extracts.

the genesis of deoxyribose. It can also be seen from Table 14 that HU suppresses the excretion of deoxyribose brought about by thymine starvation.

In order to determine whether only purine or only pyrimidine deoxynucleotides, or both, are broken down, mutants lacking either one, or both, phosphorylases were isolated in CR34 by selecting resistance to the respective deoxynucleoside. It was assumed that only purine phosphorylase and thymidine phosphorylases were likely to be involved in the production of deoxyribose-phosphates. It was argued that if only purine deoxynucleosides, or only pyrimidine deoxynucleosides were degraded, then the pup$^-$ strain or the tpp$^-$ strain, respectively, would not generate deoxyribose. If both purine and pyrimidine deoxynucleotides are degraded then both mutants should produce deoxyribose. This approach depends on the demonstration that the double mutant, pup$^-$ tpp$^-$, does not produce deoxyribose-phosphates at all.

It might be noted, first of all, that the existence of spontaneous pup$^-$ mutants which are still tlr$^-$ (see Table 24) means that purine phosphorylase is not the sole pathway for the production of deoxyribose-phosphates since, if it were, a pup$^-$ mutant would not grow on thymine at all. The results in Table 15 show that the pup$^-$ strain is induced by thymine starvation for thymidine phosphorylase,

TABLE 15. THYMIDINE PHOSPHORYLASE IN MUTANTS OF E.COLI

Strain	Genotype	Thymine concentration 20 µM[a]	1 mM[a]	Nil[b]
CR34	*tpp*$^+$ *pup*$^+$	2986	225	10,250
162B4	*tpp*$^+$ *pup*$^-$	2875	429	7,607
162-19	*tpp*$^-$ *pup*$^+$	n.d.		n.d.
162-19A17	*tpp*$^-$ *pup*$^-$	n.d.		n.d.

n.d. = not detectable

[a] Cultures were grown to 2 x 10^8/ml in glucose minimal medium supplemented with the concentration of thymine indicated. Cultures were then harvested and enzyme assayed in sonic extracts. Activities are expressed in units/mg protein.

[b] Cultures were grown in glucose minimal medium supplemented with 0.4% casamino acids and 1 mM thymine to a cell concentration of 2 x 10^8/ml and then filtered, washed and resuspended without thymine. After 3 hr the cells were harvested and treated as before.

also suggesting that deoxyribose-phosphates do not solely arise via purine phosphorylase.

The induction pattern of *tpp*$^-$ and *pup*$^-$ *tpp*$^-$ mutants is shown in Tables 16 and 17. The outstanding observation is that the double mutant is induced for phosphodeoxyribomutase when the cells are thymine starved (Table 16). This suggests that neither of the phosphorylases is the main source of dRib-5-P. (Evidence against this result being due to "leakiness" of either mutation is given later). However the *tpp*$^-$ mutant and the double mutant strain, when grown on low (20 μM) thymidine concentrations has the repressed level of mutase when compared with the levels in the parental (*tpp*$^+$) strain grown on low (20 μM) thymine or high (1 mM) thymine concentrations (Table 16). Similarly, the *tpp*$^-$ mutant has the repressed level of purine phosphorylase (Table 17). This may indicate that some dRib-5-P originates via thymidine phosphorylase, induction only being observed under conditions of thymidine starvation, i.e. when the TTP pool is reduced to zero. As discussed previously, however, it is possible that the TTP pool is high, and may be even maximal, whatever the thymidine concentration. This should result in repressed levels of enzyme.

The data in Table 16 indicating that neither purine nor thymidine phosphorylase is solely involved in the production of

TABLE 16. PHOSPHODEOXYRIBOMUTASE IN MUTANTS OF E.COLI

Strain	Genotype	Thymine source				Nil[b]
		Thymine[a] concentration		Thymidine[a] concentration		
		20 µM	1 mM	20 µM	1 mM	
CR34	*tpp*$^+$ *pup*$^+$	33	8			34
162B4	*tpp*$^+$ *pup*$^-$	48	8.7			41
162-19	*tpp*$^-$ *pup*$^+$			8	11.3	25
162-19A17	*tpp*$^-$ *pup*$^-$			14	12	27

[a] Cultures were grown to 2 x 10^8/ml in glucose minimal medium supplemented with the concentration of thymine or thymidine indicated. Cultures were then harvested and enzyme assayed in sonic extracts. Activities are expressed in units/mg protein.

[b] Cultures were grown in glucose minimal medium supplemented with 0.4% casamino acids and 1 mM thymine to a cell concentration of 2 x 10^8/ml and then filtered, washed and resuspended without thymine. After 3 hr the cells were harvested and treated as before.

TABLE 17. PURINE PHOSPHORYLASE IN MUTANTS OF E.COLI

Strain	Genotype	Thymine source			Nil
		Thymine concentration		Thymidine concentration	
		20 µM	1 mM	4µM	
CR34	*tpp*$^+$ *pup*$^+$	1331	174		2675
162-19	*tpp*$^-$ *pup*$^+$			170	1961
162B4	*tpp*$^+$ *pup*$^-$	n.d.			n.d.
162-19A17	*tpp*$^-$ *pup*$^-$			n.d.	n.d.

n.d. = not detectable

Experimental details as in Table 16.

dRib-5-P is reinforced by measuring deoxyribose excretion in the double mutant (*pup*$^-$ *tpp*$^-$) and the parent, CR34 (*pup*$^+$ *tpp*$^+$). Identical rates of excretion are observed in both strains. It was possible, however, that the deoxyribose measured, using the diphenylamine reagent (Burton, 1956) consisted of purine deoxynucleosides. This was ruled out by the fact that none of the excreted deoxyribose was charcoal adsorbable (see Methods).

The possibility that the behaviour of the *pup*$^-$ *tpp*$^-$ mutant is due to leakiness is unlikely for the following reasons. Firstly, no activity for either enzyme can be detected *in vitro*, even when the culture is induced by thymine starvation. This test is not conclusive since the enzyme may be unstable *in vitro*. Secondly, mutase is not induced by thymidine or by deoxyguanosine (Table 18). This constitutes the best evidence against leakiness, since it is an *in vivo* test. Both of these arguments also apply to the *pup*$^-$ mutant (Tables 15 and 18). The *tpp*$^-$ mutant (162-19) carries the same mutation in the *tpp* locus as the double mutant. Thirdly, the same behaviour is exhibited by three independent double mutants.

Against this however, it must be mentioned that a small but measurable rate of deoxyribose excretion occurs upon the addition of TdR to a cell suspension of the *pup*$^-$ *tpp*$^-$ mutant (see Fig.6). It seems unlikely, however, that this level of leakiness accounts

TABLE 18. INDUCTION OF PHOSPHODEOXYRIBOMUTASE AND THYMIDINE PHOSPHORYLASE IN MUTANTS OF E.COLI

Inducer	Phosphodeoxyribomutase	Thymidine phosphorylase	
	Strain: 162-19A17	162	162B4
GdR	10	6340	630
TdR	12	16070	
UdR	19		
Nil	12		637

Cultures were grown on high thymine and induced for two generations. Inducer was added to a concentration of 2 mM at the beginning of each generation. Enzyme was assayed in sonic extracts. Values are expressed in units/mg protein.

for the observed results.

It therefore seems that neither purine nor thymidine phosphorylases are solely involved in the generation of deoxyribose phosphates and an alternative route must exist. Two alternative routes were considered. Firstly, a direct production of dRib-5-P may arise via hydrolysis of the phosphodiester link of deoxynucleotides. If this was the case then a dra$^-$ drm$^-$ strain should be inducible for thymidine phosphorylase when thymine starved (and possibly when grown on low thymine concentrations). It can be seen from the experiment shown in Table 19 that this is not the case, and therefore dRib-5-P must arise via deoxynucleosides.

Secondly, in view of the latter conclusion, there must exist another enzyme, in addition to purine and thymidine phosphorylase, capable of degrading deoxynucleosides. Such an enzyme is uridine phosphorylase. When this enzyme was originally purified from E.coli its activity with deoxyuridine as substrate was not tested (Page & Schlenk, 1952). Work on the enzyme from mammalian sources has indicated that the enzyme is non-specific with respect to uridine and deoxyuridine, the ratio of uridine cleaving : deoxyuridine cleaving activity being about two (Pontis et al, 1961; Krenitsky et al, 1964). The studies by Krenitsky et al primarily directed at the mouse enzyme, also included the E.coli enzyme for comparison. It was shown that the E.coli

TABLE 19. THYMIDINE PHOSPHORYLASE IN A *dra*⁻ *drm*⁻ MUTANT (162-8) WHEN GROWN UNDER VARIOUS CONDITIONS

Culture	Thymidine phosphorylase
A	133
B	290
C	260

Culture A was grown on 20 μM thymine. Culture B was induced for two generations with thymidine (2 mM added at the beginning of each generation). Culture C was thymine starved for three hours. During this time cell mass continued normally and in parallel to a control culture of CR34, also thymine starved. The slightly higher enzyme levels in cultures B and C are probably due to some leakiness of the *drm*⁻ mutation.

enzyme was also partially non-specific, the ratio of uridine : deoxyuridine cleaving activity being about 8.6:1. The difference between the mammalian enzyme and the E.coli enzyme is thus only quantitative, despite a tendency in the literature to assume a qualitative difference (e.g. Pontis et al, 1961).

If uridine phosphorylase is involved, then the pup$^-$ tpp$^-$ mutant should be inducible by deoxyuridine. Although the level of induction is not very high, some induction of mutase is observed (see Table 18). Furthermore, if deoxyuridine is added to a cell suspension of a tpp$^-$ mutant, an appreciable amount of deoxyribose is excreted (Fig. 6). A small amount of deoxyribose is also excreted from TdR, perhaps indicating some leakiness of the mutant as mentioned above.

It is interesting that Fangman (1969) has recently reported that, in contrast to thymidine, 5-bromodeoxyuridine is rapidly degraded by a growing culture of a tpp$^-$ mutant. He suggests that another enzyme may exist for the degradation of bromodeoxyuridine. Such an enzyme may be uridine phosphorylase. He also suggests that any residual thymidine phosphorylase in his strain may have an altered affinity for thymidine and bromodeoxyuridine, the affinity for the latter being greater than for thymidine. This latter hypothesis may conceivably apply to the present situation.

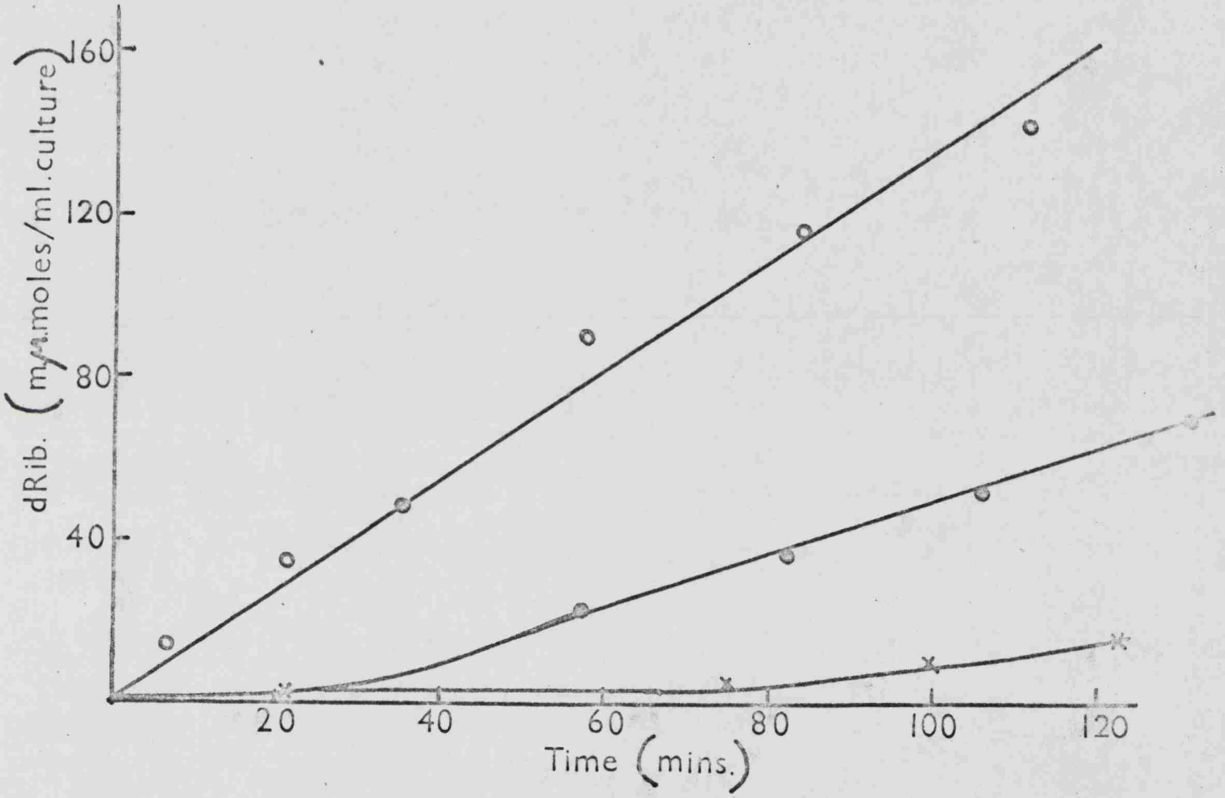
Fig. 6. Excretion of Deoxyribose from Thymidine or Deoxyuridine in Various Mutants

Exponential cultures were filtered, washed, and resuspended in M9 salts with thymidine or deoxyuridine (1 mM). Samples were taken at intervals, filtered and deoxyribose determined. In the absence of a deoxynucleoside, no excretion of deoxyribose was observed.

O = P152 (*dra*$^+$ *drm*$^-$ *pup*$^+$ *tpp*$^+$), 1 mM thymidine

X = 162-19A17 (*dra*$^-$ *drm*$^+$ *pup*$^-$ *tpp*$^-$), 1 mM thymidine

● = 162-19A17, 1 mM deoxyuridine


dRib. (mμmoles/ml.culture)
160
120
80
40
20
40
60
80
100
120
Time (mins.)

Conclusive evidence for the involvement or non-involvement of uridine phosphorylase must await the isolation of a uridine phosphorylase negative pup$^-$ tpp$^-$ mutant. Should uridine phosphorylase indeed be involved, then it may be significant that it is an intracellular enzyme (Heppel, 1967), in contrast to purine and thymidine phosphorylase (Kammen, 1967a; Munch-Petersen, 1968b). This is discussed later.

CHAPTER IV

Growth Inhibition by Deoxyribonucleosides of dra Strains

Alikhanian et al (1966) first noted that some mutants selected for their ability to grow on low external thymine concentrations have the additional property of being inhibited by thymidine. Such mutants were termed tds (thymidine sensitive) by these authors, and, like the tlr locus, were also found to map close to the thr locus. Genetic analysis has since revealed that thymidine sensitivity is a pleiotropic effect of the tlr locus. That is, the tds and tlr loci are identical (Beacham et al, 1968a).

As mentioned above, only dra$^-$ and not drm$^-$ strains are thymidine sensitive.

Pattern of Inhibition by Thymidine

The typical response to thymidine of a growing culture may be seen in Figure 7-9. There may be an initial lag of between 30-60 minutes followed by a sharp reduction or total cessation of growth. The exact response depends on the concentration of TdR used. At low TdR concentrations, recovery from the inhibition is observed. This is probably due to depletion of the thymidine added, since it is broken down by thymidine phosphorylase and excreted as deoxyribose (Barth, 1968). 20 μM thymidine is sufficient for maximum inhibition in liquid culture. The degree of inhibition, and time of recovery, are very dependent on both cell concentration and TdR

concentration. The effect of cell concentration is shown in Figure 7. At initial cell concentrations greater than 5×10^7/ml, inhibition is less marked and recovery may occur earlier. For this reason, starting cell concentrations of less than 5×10^7/ml have been employed in the majority of experiments. Since at these cell titres optical density measurements are not very reliable, cell number has been measured using a Coulter particle counter.

As expected from the occurrence of recovery from inhibition, removal of TdR from an inhibited culture leads to an immediate resumption of growth confirming that TdR is bacteriostatic rather than bacteriocidal (Fig. 8).

All deoxyribosides are inhibitory, purine deoxynucleosides being less effective than pyrimidine deoxynucleosides (see Fig. 10) at equivalent concentrations. Deoxynucleoside sensitive strains of S.typhimurium are also sensitive to deoxyribose.

The Toxic Compound

The fact that only deoxyriboaldolase negative strains are sensitive to thymidine suggests that sensitivity is due, directly or indirectly, to an accumulation of dRib-5-P in these mutants as a result of the phosphorolysis of added deoxynucleosides. It does not follow, however, that the toxic compound is also dRib-5-P.

Fig.7. Effect of Cell Concentration on Thymidine Inhibition

An exponential culture of KSU 2456 was diluted into fresh medium supplemented with TdR (60 μM) to give cell titres of 10^6 (Δ); 10^7 (○); 6 x 10^7 (●, middle curve); and 1.2 x 10^8 (●, top curve) per ml. As a control the same culture was diluted to give 10^7 cells/ml into the same medium without TdR (●, bottom curve). The slope of the control curve is reproduced alongside the two upper curves for comparison (dashed lines). Note that the right hand ordinate refers to curve (Δ) only.

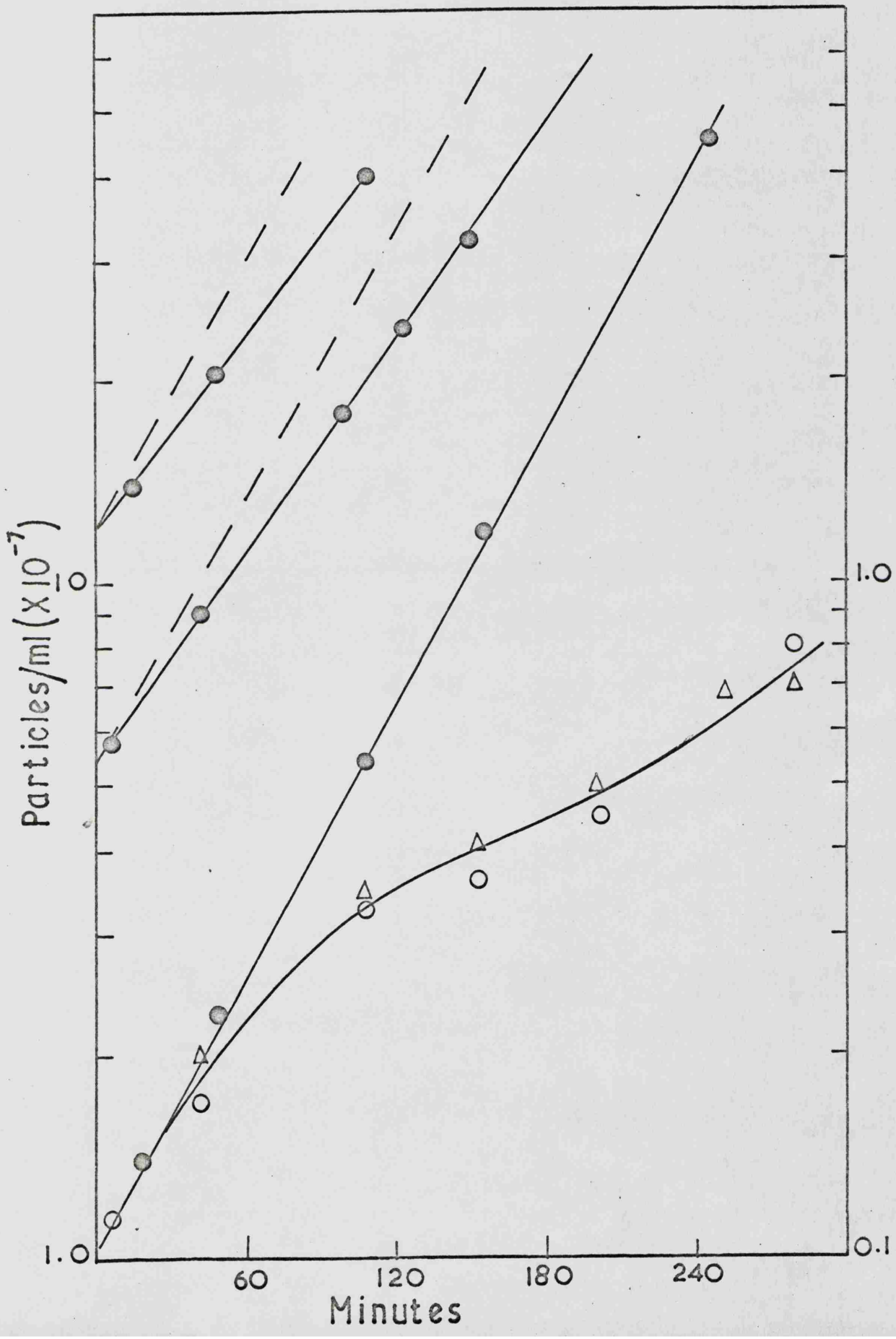
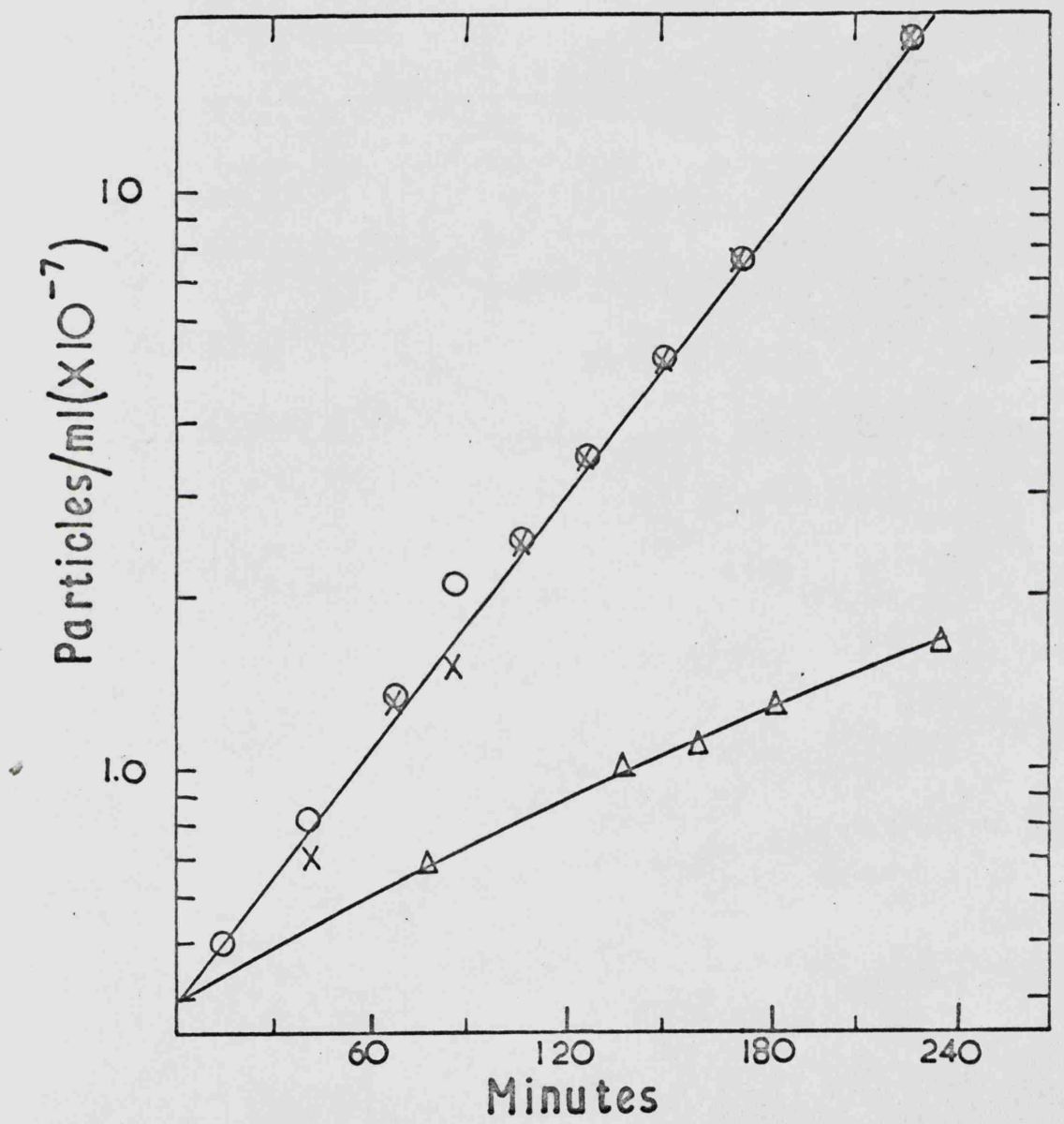

Particles/ml (X 10⁻⁷)
1.0
1.0
0.1
60
120
180
240
Minutes

Fig. 8. Growth of Inhibited Cells after the Removal of Thymidine

An exponential culture of KSU 2456 was diluted into fresh M9 medium containing 100 μM TdR and after 2.5 hr, to allow complete inhibition of growth, a portion was filtered into fresh medium containing 100 μM TdR (△) and no TdR (O). A control culture was also filtered into TdR-free medium (X).


Particles/ml(X10⁻⁷)
10
1.0
60
120
180
240
Minutes

Thus thymidine phosphorylase is an inducible enzyme and, as seen in Tables 5-7, the specific activity of thymidine phosphorylase is less in drm⁻ strains than in dra⁻ strains, particularly in E.coli. Also, tlr mutants excrete up to 80% of the deoxyribose moiety of added TdR into the medium as the free sugar (Barth, 1968). It follows that the pool size of dRib-1-P (which will be determined by the rate of input from TdR and exit as free sugar) might be lower in drm⁻ strains than in dra⁻ strains. Thus in dra⁻ strains both dRib-1-P and dRib-5-P might reach toxic levels, while in drm⁻ strains neither might do so. It cannot therefore be distinguished whether only one or both of these compounds is toxic.

Reversal of Inhibition by Ribonucleosides

Reversal of thymidine inhibition by ribonucleosides was reported by Alikhanian et al (1966). This observation has been confirmed; a five-fold excess of uridine causes complete reversal. All ribosides tested, including inosine, reverse thymidine inhibition, pyrimidine ribosides being effective at lower concentrations than purine ribosides (Fig. 9 and 10). It seemed possible that an interpretation of reversal of sensitivity by ribonucleosides might lead to an understanding of the mechanism of inhibition itself. Several alternative explanations may be considered. Firstly, there might be competition for uptake between ribosides and deoxyribosides. Secondly, ribosides might inhibit the activity, or induction,

Fig. 9. Reversal of Thymidine Inhibition by Purine and Pyrimidine Nucleosides

An exponential culture of strain 2006-1 was diluted into fresh medium containing 50 µM TdR (○), 50 µM TdR plus 250 µM GR (●), and 50 µM TdR plus 250 µM UR (⊗). A control culture with no nucleosides was also included (□).

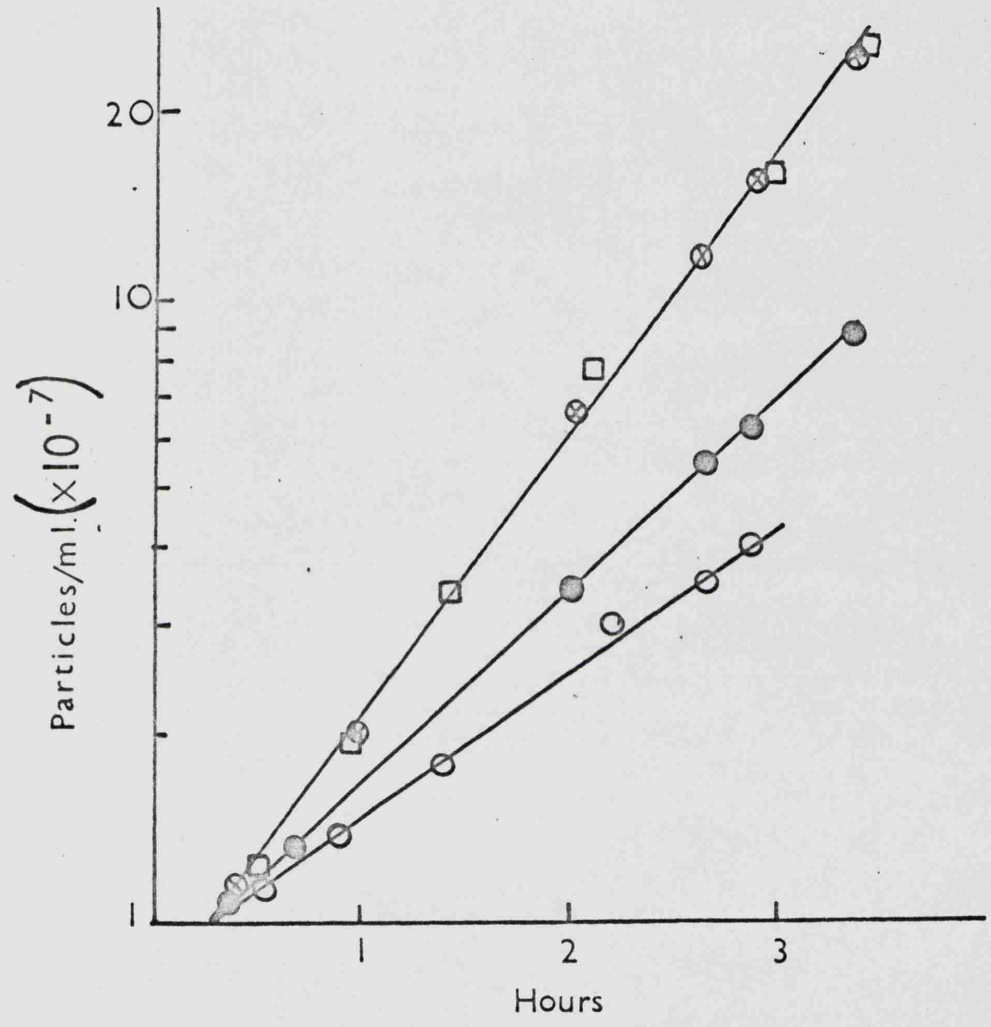
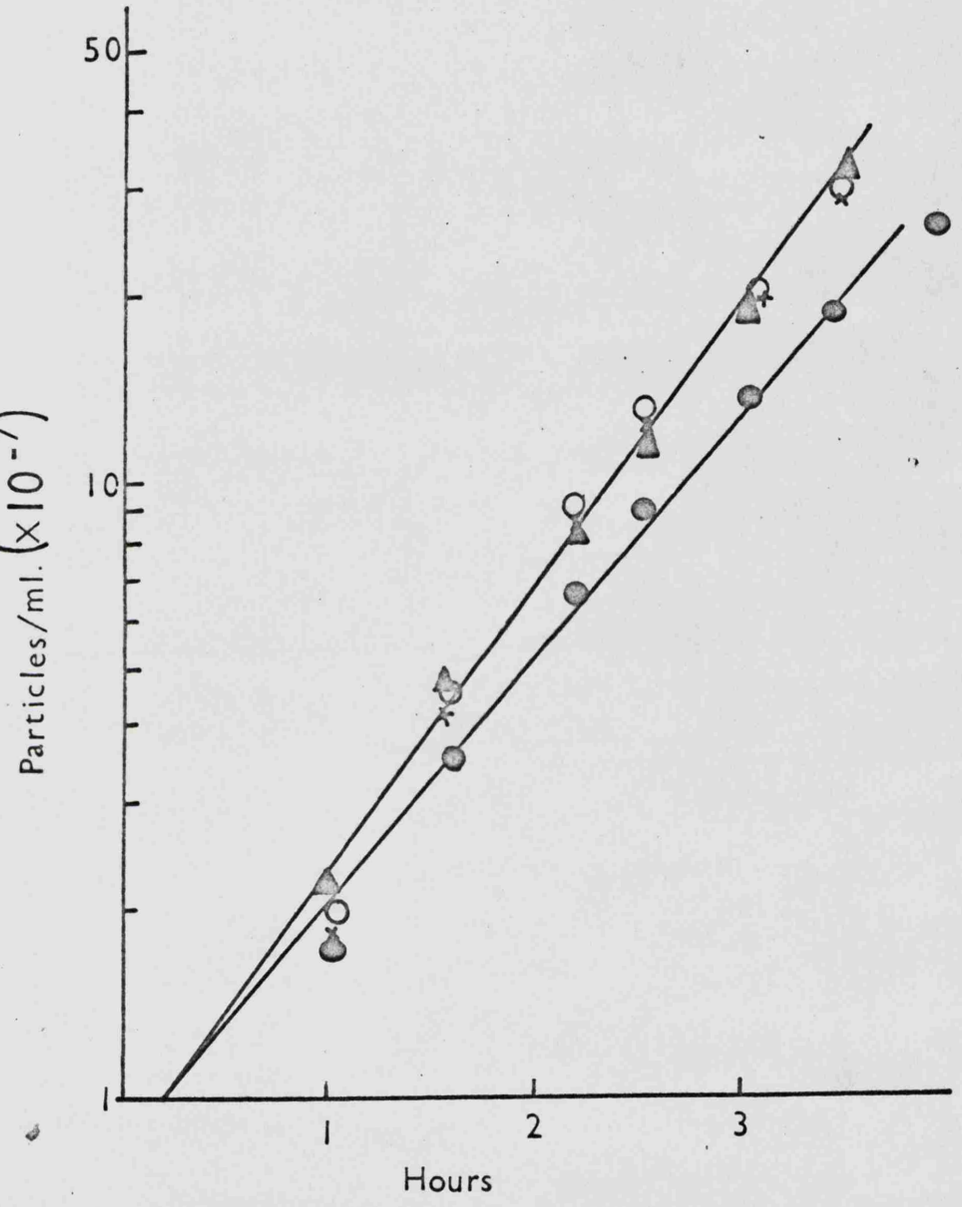

20
10
1
Particles/ml. (×10⁻⁷)
1
2
3
Hours

Fig. 10. Reversal of Deoxyguanosine Inhibition by Purine and pyrimidine nucleosides

An exponential culture of strain 2006-1 was diluted into fresh medium containing 400 µM GdR (●); 400 µM GdR plus 2 mM UR (X); 400 µM GdR plus 2 mM GR (O). A control culture was also included (▲).


50
10
1
Particles/ml. (X10⁻⁷)
1
2
3
Hours

of thymidine phosphorylase either directly or after conversion to Rib-1-P by a ribonucleoside phosphorylase. Thirdly, if sensitivity is due to inhibition of an essential biosynthetic reaction by one of the deoxyribose phosphates, the corresponding ribose phosphates may compete for the sensitive target.

The first possibility was tested by measuring the incorporation of labelled TdR in a non-sensitive tlr$^+$ strain (KSU 2793) in the presence and absence of uridine. No effect of uridine on the rate of incorporation of TdR was observed (Fig. 11) and competition for uptake is thus ruled out. It was hoped this experiment would also test the second possibility, that ribosides inhibit the activity or induction of thymidine phosphorylase, since if this were the case the cessation of incorporation of TdR due to its conversion to thymine by thymidine phosphorylase would be delayed and the amount of label incorporated correspondingly higher. The plateau level of incorporated label was indeed higher in the presence of uridine (Fig. 11) but the difference was small and the effect on thymidine phosphorylase *in vitro* was therefore examined. Table 20 shows that the rate of phosphorolysis of thymidine is reduced by uridine (see also Budman & Pardee, 1967) but not by guanosine, and that of deoxyguanosine is reduced by guanosine but not by uridine. This provides an adequate explanation for reversal by

Fig. 11. Effect of Uridine on ^{3}H - TdR Uptake

An exponential culture of KSU 2793 was diluted to give 10^{8} cells/ml into fresh medium (1.4 ml final volume) containing ^{3}H - TdR (20 μC; 2 mμmoles). Cultures also contained 40 mμmoles (Δ); 20 mμmoles (0); 10 mμmoles (●); or zero uridine(▲).

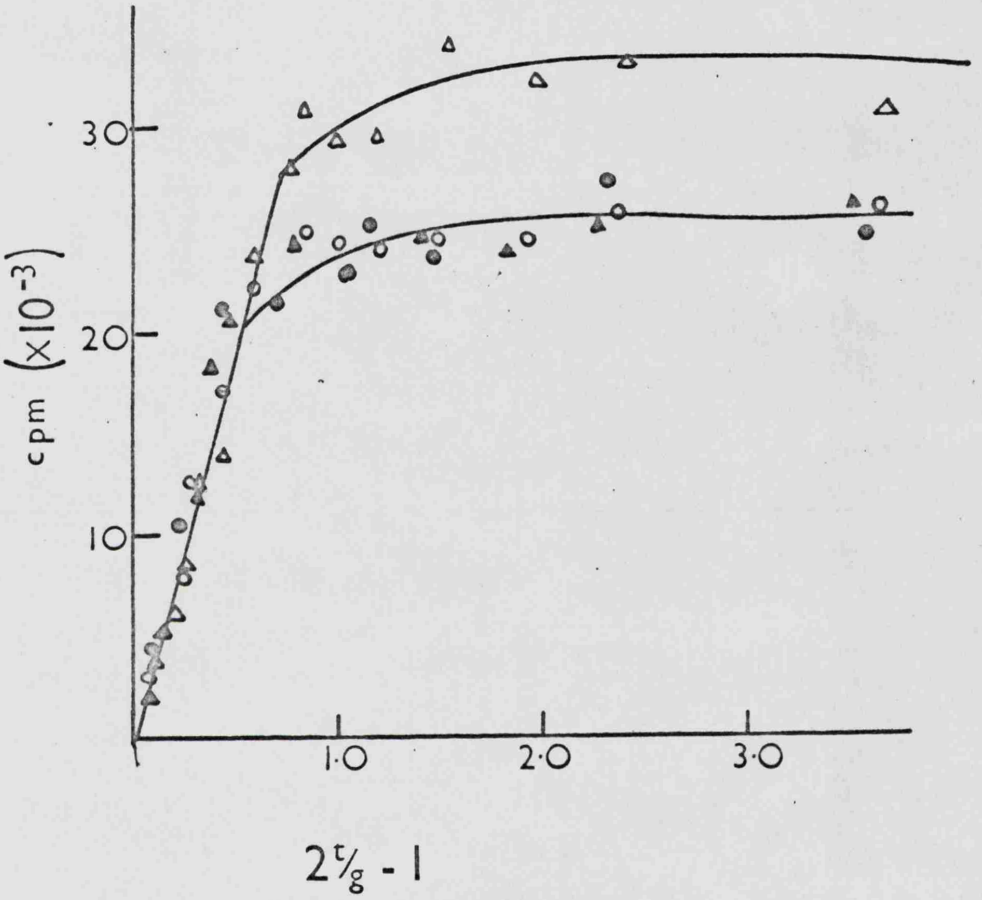

cpm (×10⁻³)
30
20
10
1·0
2·0
3·0
2^t/g - 1

TABLE 20. EFFECT OF RIBONUCLEOSIDES AND RIBOSE-1-PHOSPHATE ON THE PHOSPHOROLYSIS OF THYMIDINE AND INOSINE

Ribonucleoside	Thymidine phosphorylase		Purine phosphorylase	
	Concentration (mM)	% inhibition	Concentration (mM)	% inhibition
Uridine[a]	5	76	20	0
Guanosine[a]	10	0	0.4	46
Rib-1-P[b]	10	58	2.0	44

[a] For thymidine phosphorylase the substrate concentration was 5 mM and for purine phosphorylase the substrate concentration was 2 mM.

[b] For thymidine phosphorylase the substrate concentration was 1 mM and for purine phosphorylase, 0.4 mM.

pyrimidine ribonucleosides of inhibition of growth by pyrimidine deoxyribosides and also for reversal by purine ribonucleosides of inhibition by purine deoxyribonucleosides. The less effective reversal of pyrimidine deoxynucleoside inhibition by purine ribosides and vice versa appears not to be due to direct inhibition of phosphorylases. It is probably due either to inhibition of phosphorolysis by Rib-1-P (Table 20) produced by phosphorolysis of the ribonucleosides, or to the production of the free base, by the same mechanism, which would tend to reduce breakdown of deoxyribosides by a mass action effect. Some reversal of inhibition by the base uracil has been found (Beacham et al, 1968a).

Thus although other explanations are not ruled out, it seems probable that reversal is due to a reduction in the rate of production of deoxyribose phosphates, rather than to competition between ribose phosphates and deoxyribophosphates for the sensitive target.

The Nature of Thymidine Inhibition

In an attempt to define more closely the nature of the inhibitory effect of thymidine, the kinetics of inhibition of RNA and DNA synthesis were followed (Fig. 12 and 13). RNA and DNA synthesis were determined by measuring incorporation of ^{32}P.

Fig. 12. Effect of Thymidine on RNA Synthesis

An exponential culture of KSU 2456 was diluted into fresh low-phosphate medium (Kjeldgaard, 1961) containing $^{32}PO_4$ (0.3 μc/ml). 1 ml samples were removed at intervals and assayed for $^{32}PO_4$ incorporation into RNA. (O) 6 μM TdR; (●) No TdR. The doubling time (g) was 54 min.

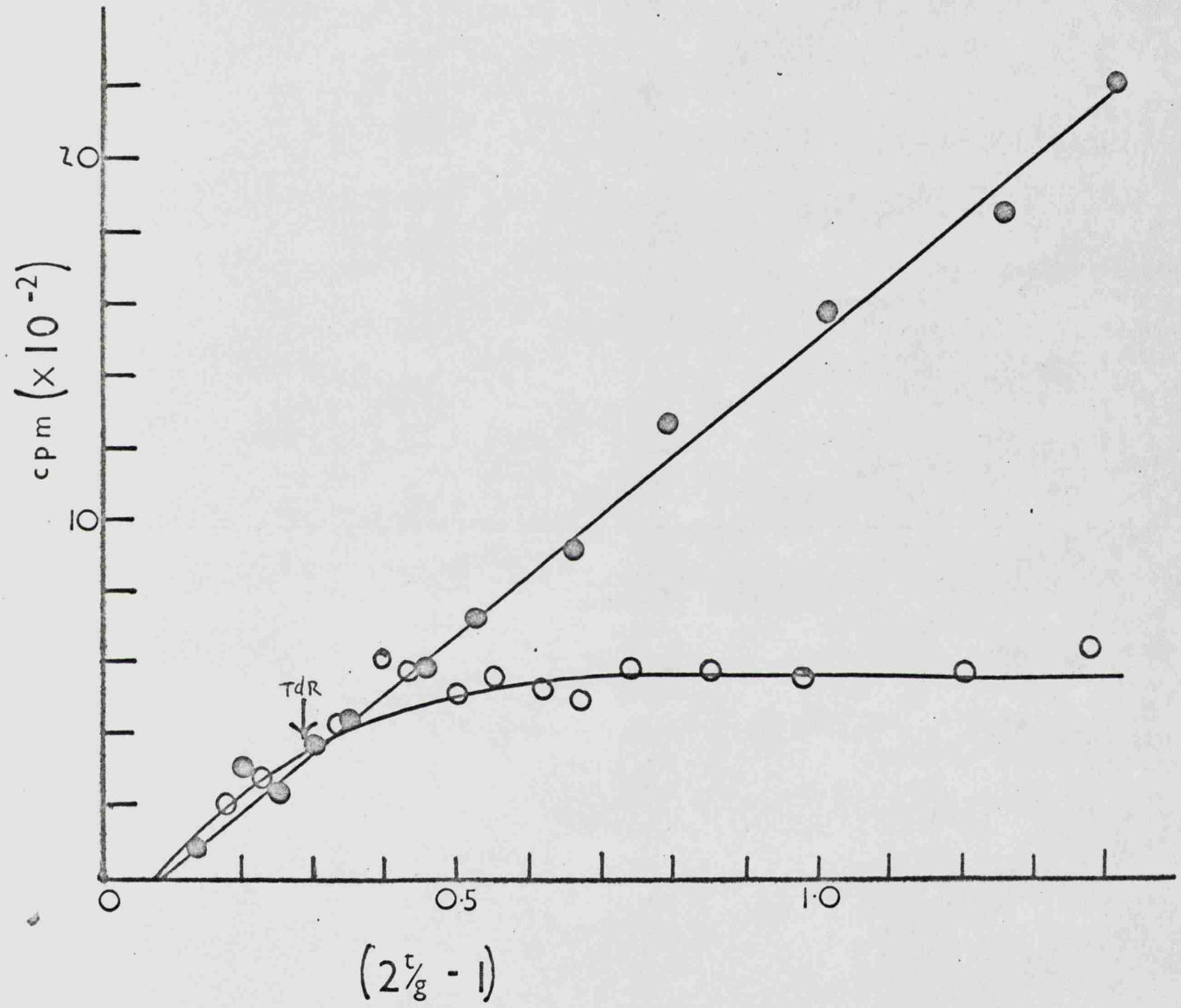
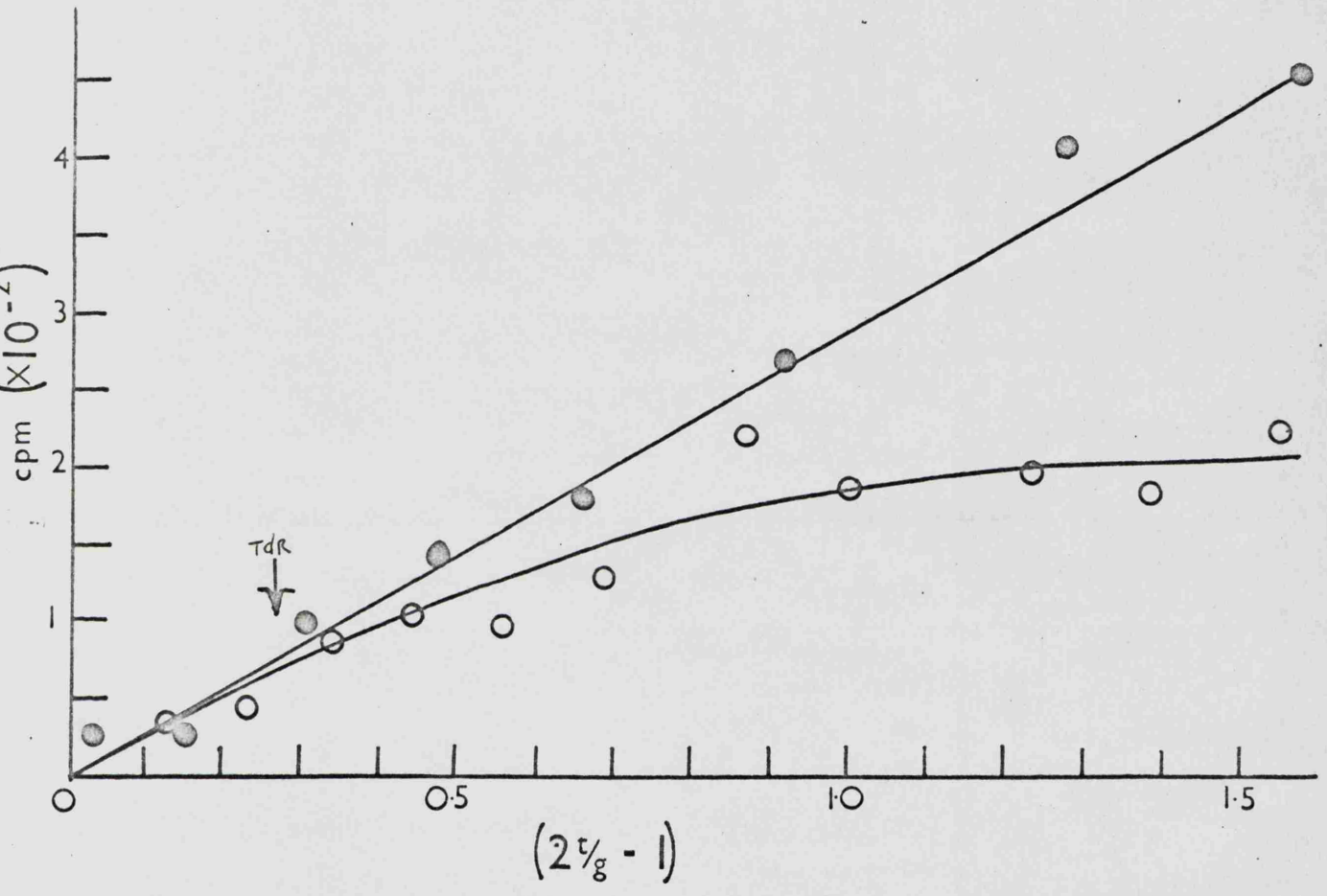

20
10
cpm (×10⁻²)
TdR
0
0·5
1·0
(2^t/g - 1)

Fig. 13. Effect of Thymidine on DNA Synthesis

In the experiment described in Fig.12, samples were also assayed for $^{32}PO_4$ incorporation into DNA (see Methods). (O) 60 μM TdR; (●) No TdR.


cpm (×10⁻²)
4
3
2
1
0
TdR
0·5
1·0
1·5
(2t/g - 1)

A marked inhibition of RNA synthesis occurs within 6 minutes of addition of thymidine. The rate of DNA synthesis, on the other hand, is not appreciably altered for about 20-30 minutes. This was confirmed by measuring ^{3}H-thymine incorporation in KSU 2454 using deoxyuridine as inhibitor (Fig. 14). It seems that the primary effect of thymidine is not on DNA synthesis.

The effect on respiration was also determined using an oxygen electrode and non-growing cells (Fig. 15). It can be seen that the addition of TdR brings about an immediate reduction in the rate of oxidation of glucose. This result is consistent with a primary effect of deoxynucleosides on respiration, and agrees with the results of Lomax and Greenberg (1968). However, the inhibitory effect of thymidine is also seen in cells growing anaerobically (Table 21). These results are discussed later.

In view of the evidence that dRib-5-P might be the inhibitory compound, its effect on the metabolism of Rib-5-P via the pentose phosphate cycle was determined *in vitro*. In sonic extracts from KSU 2454 and 2456 the rate of Rib-5-P-dependent reduction of NADP was reduced by approximately 30% by dRib-5-P when the molar concentration of the latter was in eight-fold excess of the former (Table 22). The data are thus consistent with the possibility that there is interference with the pentose phosphate cycle but, in the

Fig.14. Effect of Deoxyuridine on ^{3}H-Thymine Incorporation

An exponential culture of KSU 2454 was diluted to a cell concentration of 10^{7}/ml into glucose M9 medium containing ^{3}H-thymine (12.5 μC/mμmole). The total thymine concentration, in both cultures, was 16 μM. Deoxyuridine (200 μM) was added to one culture (O) at the time indicated by the arrow. The other culture (■) contained no deoxyuridine. Samples were removed at intervals and incorporation determined as described in Methods. g = 48 min.

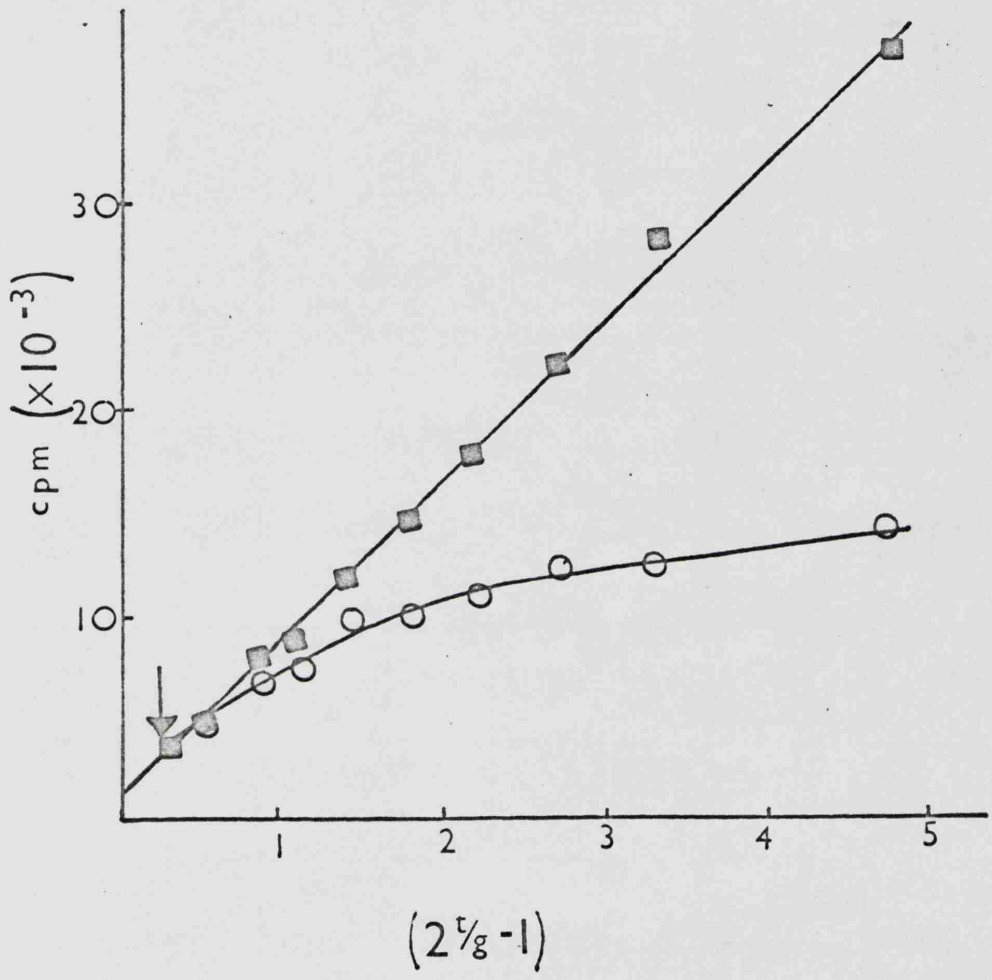

cpm (×10⁻³)
30
20
10
1
2
3
4
5
(2^{t/g} - 1)

Fig. 15. Effect of Thymidine on Respriation in Resting Cells of E.coli CR34

Respiration was measured as described in Methods. Curve 'a': a control without thymidine; curve 'b': thymidine was added at the time indicated by the arrow; curve 'c': thymidine was present from time zero.

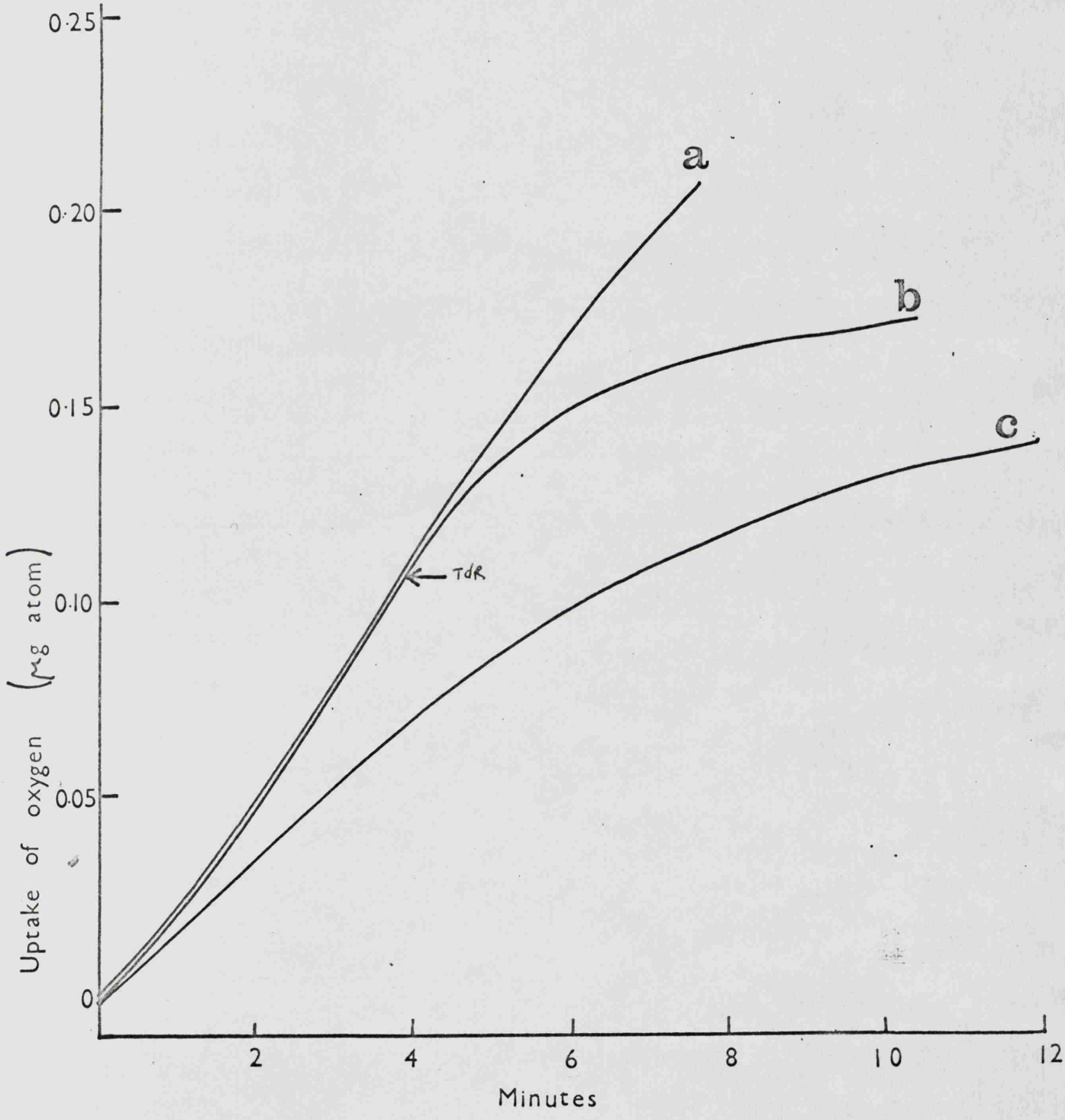

0·25
0·20
0·15
0·10
0·05
0
Uptake of oxygen (mg atom)
a
b
c
TdR
2
4
6
8
10
12
Minutes

TABLE 21. GROWTH INHIBITION BY THYMIDINE OF *dra*⁻ STRAINS UNDER ANAEROBIC CONDITIONS

Time	Tube No.	1	2	3	4	5	6
	Thymidine:	+	+	-	-	+	-
4 hr		.061		.25		0.061	0.256
20 hr			.059		.97	0.151	1.08

Values refer to the absorbance at 600 mμ. *E.coli* CR34 was grown to 2×10^8 cells/ml and diluted into fresh medium with or without thymidine (1 mM), contained in sterile Thunberg tubes. The cell titre was 10^7/ml and the A_{600} 0.054 at time zero. Tubes 1-4 were then evacuated, flushed with argon, and finally filled with argon. At the times indicated the absorbance at 600 mμ was determined.

TABLE 22. EFFECT OF DEOXYRIBOSE-5-PHOSPHATE ON OVERALL TRANSKETOLASE-TRANSALDOLASE ACTIVITY IN EXTRACTS OF S.TYPHIMURIUM

Strain	Ribose-5-phosphate	Deoxyribose-5-phosphate	Specific activity[a]
2456	0.5 µmoles	-	8.54
	0.5 µmoles	0.6 µmoles	9.34
	0.5 µmoles	3.85 µmoles	5.84
2454	0.5 µmoles	-	12.40
	0.5 µmoles	3.85 µmoles	9.11

[a] mµmoles NADPH formed/min/mg

Activity was determined in sonic extracts as described in Methods.

Deoxyribose-5-phosphate was included in the reaction mixture as indicated above.

absence of data on the internal pool sizes of Rib-1-P and dRib-5-P, it is not possible to calculate whether the degree of inhibition observed is sufficient to account for the toxic effect of thymidine. The fact that some *in vitro* inhibition of transketolase-transaldolase activity is observed and that ribose does not reverse the inhibition, even when used as sole carbon source, suggests that if sensitivity is due to an effect on the pentose-phosphate cycle, then the transketolase-transaldolase reactions are the ones involved. However, it is not clear why a partial inhibition of this pathway should have the marked effect on growth that we have observed, particularly since it has been estimated that only about 30% of glucose catabolism proceeds via the pentose-phosphate cycle (Wang *et al*, 1958) and only a minor proportion of Rib-5-P is used in nucleic acid synthesis (Katz & Rognstad, 1967). It is possible, however, that the inhibition of transketolase-transaldolase may be much greater *in vivo*.

Ribose-5-P is also involved in the synthesis of PRPP via 5-phosphoribose pyrophosphokinase. However, it has been reported that dRib-5-P does not inhibit this enzyme (Flaks, 1963).

Two other specific hypotheses have been considered. Firstly, that high intracellular levels of dRib-5-P might feedback inhibit the uptake of inorganic phosphate. The uptake of phosphate is

almost certainly an active process (Damadian, 1967; Rosenberg, 1968). A prediction of this hypothesis might be that severely inhibited cells would be starved of inorganic phosphate. This should have two consequences: a derepression of alkaline phosphatase (Torriani, 1960) and breakdown of ribosomal RNA (Maruyama & Mizuno, 1956). However, I have found that there is no release of isotope from cells prelabelled with ^{3}H-uridine and then severely inhibited by a high concentration of thymidine. The data in Table 23 shows there is also no change in the level of alkaline phosphatase. A deficiency of phosphate is therefore considered to be unlikely as a cause of thymidine inhibition.

Secondly, the uptake of glucose might be similarly inhibited, perhaps via the phosphotransferase system (Simoni et al, 1964). The fact that deoxynucleoside sensitivity is also observed on a variety of other carbon sources (succinate, glycerol, ribose, pyruvate, xylose,mannitol gluconate) need not necessarily be inconsistent with this hypothesis since mutations in the phosphotransferase system are strongly pleiotropic, i.e. the uptake of a wide variety of carbohydrates is affected (Simoni, et al, 1964; Wang & Morse, 1968). However, sensitivity is still observed in casamino acids medium and also in tryptone and nutrient broth

TABLE 23. ALKALINE PHOSPHATASE DURING THYMIDINE INHIBITION

Time after addition of TdR	Alkaline phosphatase units/A_{600}
0	0.24
40	0.32
90	0.24

E.coli CR34 was grown in a Tris buffered medium containing inorganic phosphate 0.5 mM. Thymidine (2 mM) was added to an exponentially growing culture at a cell titre of 2×10^7/ml. Complete inhibition of growth occurred within 15 min. Samples (10 ml) were taken before and after the addition of thymidine, centrifuged, washed, resuspended in Tris buffer (1 M), pH 8.0, and alkaline phosphatase assayed. A control culture, deprived of inorganic phosphate for 60 min, and treated identically contained 40 units/A_{600} unit.

medium. Inhibition of growth is less marked in the latter two media but very marked in casamino acids (see Fig. 16 and 17).

It is also possible that the toxic effect of deoxyribose phosphates is related to the numerous other cases in E.coli of sugar toxicity in mutants which cannot metabolise phosphorylated derivatives of these sugars (see Bock & Neidhardt, 1966a,b). This will be fully discussed later.

Thymidine Resistant Mutants

From Figure 1 it is clear that secondary mutants from a dra$^-$ strain lacking any enzyme needed for the formation of dRib-5-P from a deoxynucleoside, should be resistant to that deoxynucleoside. Resistant mutants are in fact readily obtained by plating 10^7-10^8 cells on glycerol minimal agar plates containing 2 mM deoxynucleoside. Resistant clones arise with high frequency in 24-36 hours. Glycerol was used as a carbon source since less recovery of the background cells occurs than when glucose is used. The reason for this is not clear. High concentrations of deoxynucleoside were used also to reduce recovery from inhibition.

When TdR was used the resistant mutants are found to fall into two main classes (see Table 24). The first class will not grow on thymine (400 µm) at all, and the second class, which retain this ability, are still tlr$^-$. The first class are thymidine

Fig.16. Thymidine Sensitivity in Nutrient Broth

Cells were diluted from exponential growth in nutrient broth to fresh nutrient broth medium containing either 1 mM thymidine (O) or no thymidine (●). Almost identical results were obtained in tryptone medium.

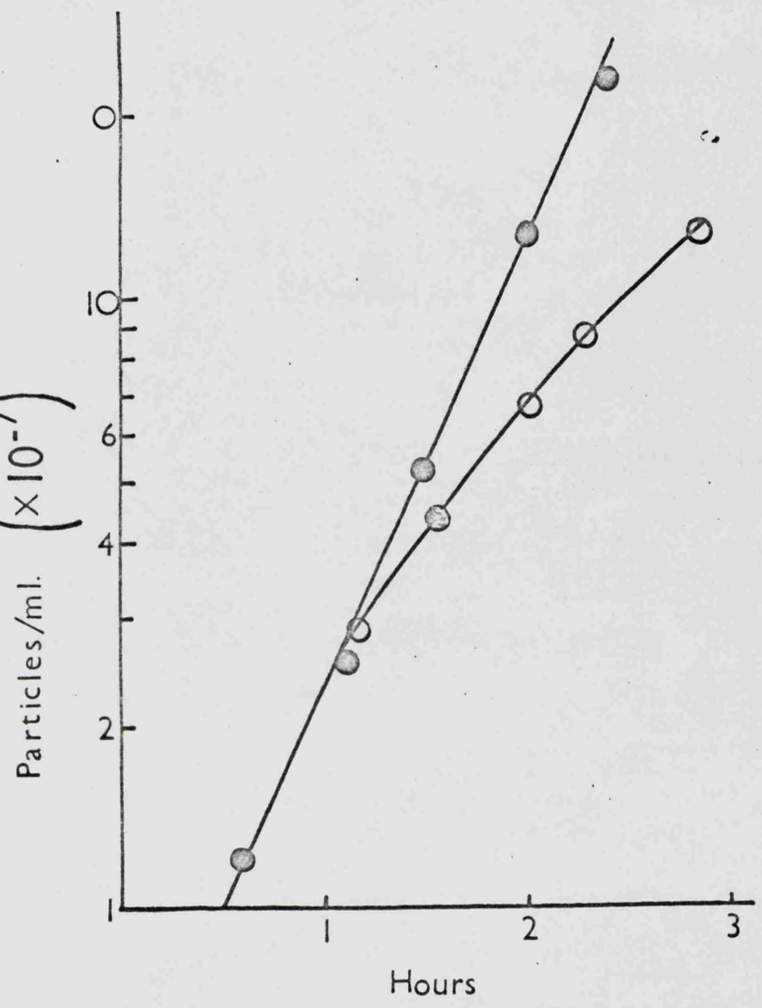

Particles/ml. (×10⁻⁷)
0
10
6
4
2
1
1
2
3
Hours

Fig. 17. Thymidine Sensitivity in M9-Medium Supplemented with 1% Casamino Acids

Cells were diluted from exponential growth in a casamino acids medium into the same medium containing 1 mM thymidine (O)or no thymidine (●).

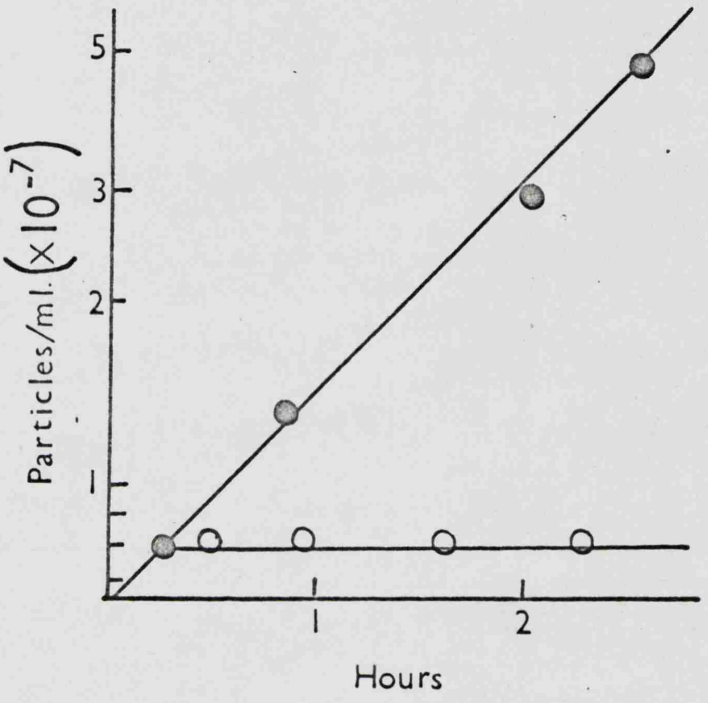

Particles/ml. (×10⁻⁷)
5
3
2
1
1
2
Hours

TABLE 24. PROPERTIES OF DEOYRIBONUCLEOSIDE RESISTANT MUTANTS OF *E.COLI* CR34

Mutants isolated as resistant to:	Class No.	Sensitivity to: TdR	GdR	Growth on: UR	GR	Ability to use thymine as a thymidylate source: 20 µM	400 µM	Inferred genotype
TdR	1	-	+	+	+	-	-	*dra*$^-$ *drm*$^+$ *tpp*$^-$ *pup*$^+$
	2	-	-	-	-	+	+	*dra*$^-$ *drm*$^-$ *tpp*$^+$ *pup*$^+$
	3	-	-	+	+	-	+	*dra*$^+$ *drm*$^+$ *tpp*$^+$ *pup*$^+$
GdR	1	+	-	+	-	+	+	*dra*$^-$ *drm*$^+$ *tpp*$^+$ *pup*$^-$
	2	-	-	-	-	+	+	*dra*$^-$ *drm*$^-$ *tpp*$^+$ *pup*$^+$
	3	-	-	+	+	-	+	*dra*$^+$ *drm*$^+$ *tpp*$^+$ *pup*$^+$

Sensitivity to deoxynucleosides was scored after overnight incubation on plates containing 1 mM deoxynucleoside; - = resistant, + = sensitive. For growth on nucleosides as a sole carbon source, a 5 mM concentration was used.

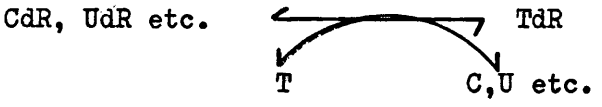
phosphorylase negative (tpp$^-$) and require thymidine for growth; the second class are drm$^-$. Similarly when deoxyguanosine (plus 400 µm thymine) was used, two main classes of mutants arose. The first class are unable to grow on uridine as sole carbon source whereas class 2 are able to. The former are drm$^-$ and the latter purine phosphorylase negative (pup$^-$). Neither class grew on guanosine as sole carbon source and both classes are tlr$^-$. Lack of growth on guanosine is consistent with the fact that purine phosphorylase is non-specific with respect to ribo- and not deoxyribo- nucleosides (Ahmad et al, 1968; Karlstrom, 1968). Using both TdR and GdR, a rare third class was found (see Table 24) which are assumed to be dra$^+$ revertants. The mutants described above have no detectable enzymatic activity at all for the relevant defective enzyme. The method is therefore veryuseful for the selection of phosphorylase negative mutants (see also Hoffee & Robertson, 1969).

It was hoped that by using deoxycytidine as the inhibitory deoxynucleoside, cytidine deaminase negative strains (cdd$^-$) would also be present (see Fig.1). Such mutants should grow on uridine as a sole carbon source but not on cytidine, and should still be sensitive to deoxyuridine. However, in 200 mutants examined none were found. Cdd$^-$ mutants are readily isolated, however, by selecting for resistance to fluorodeoxycytidine (Karlstrom, 1968). Such

mutants have the expected properties demonstrating that the screening tests are valid. The reason for the extreme rarity, or absence, of *cdd*$^-$ mutants among deoxycytidine resistant mutants is not known.

The existence of the various classes of resistant mutant described above allows two conclusions to be made. Firstly, since resistant mutants are blocked in the catabolism of deoxynucleosides, it is catabolism and not anabolism of deoxynucleosides that leads to sensitivity. Secondly, the inability of *tpp*$^-$ mutants to grow on thymine demonstrates that thymidine phosphorylase is necessary for thymine uptake and is the main pathway for the conversion of thymine to thymidine. The *thy*$^+$ *tpp*$^-$ mutant of Fangman and Novick (1966) is also unable to utilise thymine in the presence of deoxyadenosine (Novick, quoted by Denhardt, 1969; Budman & Pardee, 1967).

Another possible route for the uptake of thymine, however, is via deoxyribosyl-transferases, viz:



The status of deoxyribosyl-transferases, and their possible role in thymine incorporation, is discussed in full later. It may be noted here, however, that the isolation of *tpp*$^-$ and *tpp*$^-$ *pup*$^-$ mutants allows a simple test to be made of whether such a route

for the incorporation of thymine is possible. Thus although thy⁻ tpp⁻ mutants are unable to grow even with very high thymine concentrations (1 mM), they should do so in the presence of a deoxynucleoside if TdR can be formed via a deoxyribosyl-transferase reaction. It is found that strain 162-19 is able to grow in the presence of AdR, GdR, UdR or CdR and 1 mM thymine. No such effect is observed with a low (40 µM) concentration of thymine. The ability to grow with AdR or GdR and thymine is lost in the double mutant pup⁻ tpp⁻, but the effect of CdR and UdR remains. Although these results could theoretically be explained by 'leakiness' of the mutations involved, reasons for ruling this out have been given. Most likely the effects of deoxynucleoside are due to deoxyribosyl transferase activity. The loss of growth on thymine with AdR or GdR, which is concurrent with loss of purine phosphorylase activity, may be explained by a slight purine-pyrimidine deoxyribosyl transferase activity being associated with the purine phosphorylase enzyme. Such activity is associated with thymidine phosphorylase (Vilstrup, quoted in Munch-Petersen, 1968b; Mineeva & Il'ina, 1969). The effect of CdR and UdR in both the tpp⁻ and pup⁻ tpp⁻ mutant is probably due to pyrimidine specific deoxyribosyl transferase (Lampen, 1952).

Another feature of tpp⁻ pup⁻ strains is that their growth

is essentially unimpaired by the presence of high concentrations (2 mM) of AdR or TdR. This is in contrast to the case with mammalian cells where AdR or TdR strongly inhibits DNA synthesis (Cooper et al, 1966; Klenow, 1959; 1962). It has been shown with mammalian cells that dATP is found to accumulate during inhibition (Munch-Petersen, 1960) by AdR, and that a cell line deficient in thymidine kinase is resistant to inhibition by TdR (Morris & Fischer, 1960). It is known that dATP and TTP are allosteric inhibitors of ribonucleotide reductase and it is reasonable to suppose that dATP (or TTP) inhibits the reductase, thereby preventing the synthesis of all four deoxynucleotides necessary for DNA synthesis (see Reichard, 1967). The lack of inhibition of AdR or TdR in E.coli cannot be due to rapid catabolism in a pup^- tpp^- mutant. In the case of AdR it is most likely to be due to a lack of phosphorylation of deoxyadenosine. Thus Neuhard (1967) has shown that in cells depleted of deoxynucleoside triphosphates by treatment with hydroxyurea, the addition of all four deoxynucleosides causes only the thymidine triphosphate pool to rise towards normal levels. This suggests that TdR is the only deoxynucleoside to be phosphorylated in E.coli. The inhibition of DNA synthesis by TdR in mammalian cells probably resides in the fact that the addition of external TdR brings about an expansion of the total TTP and TDP pool by a factor of approximately ten (Cooper et al,

1966). However, the size of the total thymine nucleotide pool in E.coli has been measured in the presence of external TMP (Dilworth-Cannon & Breitman, 1967; 1968). The data show that a 40% increase in the thymine nucleotide pool occurs and de novo synthesis of thymine nucleotides is reduced by 35%. Thus the fact that the TTP pool in mammalian cells expands by a factor of about ten, but only only by 40% in E.coli, may account for the fact that TdR inhibits DNA synthesis in the former case, but not in E.coli. The difference in the behaviour of the TTP pool in the two systems may reside in differences in any of the enzymes catalysing the conversion of TdR to TTP.

DISCUSSION

(1) Tlr Mutants: Their Nature and Properties

Although it is clear that, in general, *tlr* mutants of *E.coli* and *S.typhimurium* are *drm*$^-$, *dra*$^+$, or *drm*$^+$ *dra*$^-$ (Hoffee, 1968a; Munch-Petersen, 1968a; Breitman & Bradford, 1967b, 1968; Beacham *et al*, 1968a; Barth *et al*, 1968), there are some mutants which do not seem to fit into either of these categories or which have anomalous properties.

One of these is a *drm*$^-$ strain isolated by Okada and studied by Munch-Petersen (1968a). This strain is constitutive for deoxyriboaldolase and thymidine phosphorylase, in contrast to the majority of *drm*$^-$ mutants. It was suggested that this mutant is a deletion mutant covering the *drm* locus and an adjacent regulator locus (Ahmad & Pritchard, 1969). Since the gene order is *dra-tpp-drm-pup*, this assumes that there are two operons, and such a deletion fuses the *dra-tpp* and the *drm-pup* operon. However, this hypothesis now seems unlikely in view of the fact that the mutant is revertible for the *drm* phenotype. Also, this strain contains purine phosphorylase activity, which is specified by an adjacent gene (Ahmad, personal communication). Thus a deletion does not extend into the *pup* gene. Finally, *drm*$^+$ revertants and transductants are still constitutive for thymidine phosphorylase. It seems, therefore, that this strain is a double mutant, perhaps

containing a mutation in an "i" type gene in addition to a non-functional drm gene. There are no obvious reasons for constitutivity accompanying a mutation in the drm gene, although it is possible that this particular mutant was selected on an extremely low thymine concentration; constitutive thymidine phosphorylase synthesis (or constitutivity of any of the enzymes responsible for the production of deoxyribose phosphates) may allow a higher pool size of dRib-1-P and therefore allow growth on a very low external thymine concentration. H.O. Kammen has also isolated a constitutive drm$^-$ strain (personal communication).

Hoffee (1968a) found that tlr mutants of S.typhimurium fell into four groups. The first group were not constitutive for deoxyriboaldolase or thymidine phosphorylase; phosphodeoxyribomutase was not assayed. This group may be drm$^-$. The second group were dra$^-$ and constitutive for thymidine phosphorylase. Group three and group four mutants, however, do not lack deoxyriboaldolase, yet both are constitutive for thymidine phosphorylase. Group three mutants (but not group four) are also constitutive for deoxyriboaldolase. Both groups three and four are sensitive to deoxyribose but only group three mutants are unable to utilise deoxyribose. Hoffee accounts for the properties of the group three and group four mutants by proposing that S.typhimurium contains

two types of deoxyriboaldolase, type I and type II (Hoffee, 1968a). Her main evidence for this was two-fold: firstly, when deoxyriboaldolase is induced with deoxyribose a biphasic induction is obtained. Secondly, when mutants are selected on the basis of their inability to grow on deoxyribose, no deoxyriboaldolase negative mutants are obtained. (The *dra*$^-$ mutants found among *tlr* mutants (group 2 above) were apparently treated with a mutagen prior to the selection for *thy*$^-$ and hence may be double mutants (Hoffee, 1968a)) However, in the present investigation, mutants totally lacking deoxyriboaldolase were readily obtained among spontaneous *tlr* mutants of *S.typhimurium* (see Table 4). The reason for this discrepancy is not known.

It was initially postulated by Hoffee that type I aldolase together with deoxyribokinase, and a deoxyribose permease, was induced by deoxyribose. Thus the genes specifying these three enzymes were postulated to form an operon different to that formed by the genes for aldolase type II, thymidine phosphorylase and phosphodeoxyribomutase and induced by dRib-5-P. In a later paper, however, data on the kinetics of induction are presented which are inconsistent with dRib being the inducer of type I aldolase (Hoffee & Robertson, 1969). The second phase of induction seems to

depend on the induction of significant levels of deoxyribokinase. The authors suggest that dRib-5-P might also be the inducer of type I aldolase, but that higher concentrations are necessary than for the induction of type II aldolase. Although these observations are hard to explain on any other hypothesis, the evidence for two deoxyriboaldolases is not conclusive. A deoxyriboaldolase has been purified from wild type S.typhimurium by Hoffee (1968b) and stated to be type I; it was also stated that it is separated from type II aldolase by ammonium sulphate fractionation, but this aspect has not yet been reported in detail. The group 3 and group 4 tlr mutants are postulated by Hoffee to lack either type I or type II aldolase, respectively. However, in the case of group 3 mutants, which possess high levels of aldolase, it is not clear why they should be tlr$^-$. It is similarly difficult to understand why such mutants are unable to ferment deoxyribose and are sensitive to deoxyribose.

The group four mutants have low levels of deoxyriboaldolase, but have a high level upon induction with deoxyribose. This is consistent with the loss of one type of aldolase, although the remaining enzyme must have an induction ratio of about 500. Since these mutants do possess deoxyriboaldolase, it is again difficult to understand why they are sensitive to deoxyribose.

An alternative interpretation of the group three mutants might be that they are drm$^-$ mutants of the type described above and reported by Munch-Petersen (1968) and Kammen (personal communication). Thus both types are constitutive for deoxyribo-aldolase and thymidine phosphorylase.

Similar to the group three and group four tlr mutants of Hoffee are strain 2006-2, 2006-9 and 2006-12 described in Table 4 and termed dra^c. These tlr mutants of S.typhimurium behave in all respects like dra$^-$ mutants: they are constitutive for thymidine phosphorylase, at least two of them are constitutive for phospho-deoxyribomutase, they are sensitive to thymidine and deoxyribose, they are unable to utilise deoxyribose and, of course, they are tlr$^-$ (see Tables 4 and 5b). However, these strains undoubtedly contain deoxyriboaldolase (Table 4). Surprisingly, though, this enzyme does not appear to be constitutive. If, however, the enzyme is mutationally altered then it is possible that the in vitro activity will be very low, although the enzyme is still constitutive-ly synthesised; the result may be apparently uninduced levels of enzyme. This predicts that, when grown on high external thymine concentrations, the level of deoxyriboaldolase will be even lower in these strains, since the degree of endogenous induction is dependent on the external thymine concentration (see Chapter III;

this prediction is not dependent on any specific hypothesis with respect to the nature of the thymine concentration effect). This prediction is verified by the data in Table 25.

Two explanations are possible for this group of tlr mutants. Firstly, on the hypothesis of Hoffee (1968a), there may be two deoxyriboaldolases in S.typhimurium, and these mutants have lost one of them. As mentioned above, however, it is not likely that such mutants would display the phenotype described. Some utilisation of deoxyribose would be expected and, also, at least a rapid recovery from inhibition by thymidine and deoxyribose. Neither of these expectations are fulfilled. Furthermore, in view of the fact that almost wild-type levels of deoxyriboaldolase are present, it would not be expected that such mutants by tlr^{-}. Finally, the existence of mutants totally lacking deoxyriboaldolase would be very rare on such a hypothesis but in fact are readily obtained (Table 4).

Secondly, these mutants may be cryptic. That is, whole cells are unable to metabolise a substrate, but the enzyme is active in cell free extracts. There may be two types of crypticity which would account for the properties of dra^{c} mutants. (1) The cells are no longer permeable to the substrate; thus intracellular enzymes would not have access to the substrate. (2) In the case of surface located enzymes it is possible that the enzyme may be

TABLE 25. AFFECT OF THYMINE CONCENTRATION ON DEOXYRIBOALDOLASE IN *dra*^{c} MUTANTS OF *S.TYPHIMURIUM*

Strain	Thymine concentration	
	20 µM	800 µM
2006-2	26.6	6.55
2006-9	22.6	3.35
2006-12	21.7	10.4

Cultures were harvested in exponential phase and enzyme assayed in osmotic shock extracts by Method 2.

altered such that it can no longer hydrolyse a substrate but retains *in vitro* activity. This type of cryptic mutant has been reported by Ward and Glaser (1968) in the case of UDP-glucose hydrolase of *E.coli*. (This enzyme is identical to 5'-nucleotidase in *E.coli* (Glaser *et al*, 1967; Neu, 1967a)). Mutants probably similar to this were also isolated in the present investigation, for the same enzyme (see Section 3, Chapter II). The essential feature of the data of Ward and Glaser (1968) is that whole cells showed no UDP-glucose hydrolase activity, but activity was present in cell-free extracts. Since the enzyme is a surface enzyme (Glaser *et al*, 1967; Dvorak *et al*, 1966; Neu, 1967b) then considerations of permeability are unlikely to be relevant to this observation. Furthermore, UDP-glucose is likely to be impermeable to the cell normally; hydrolysis of UDP-glucose by whole cells is expected due to the surface location of the enzyme. Ward and Glaser (1968) proposed that this type of cryptic mutant may result from a mutation in the structural gene for the enzyme or, alternatively, in a gene specifying a protein which is necessary for the correct surface orientation and function of the enzyme. No evidence for the former alternative was found.

Although deoxyriboaldolase is released by osmotic shock in *S.typhimurium* (see Table 4), this does not necessarily imply that

the situation is analogous to that for UDP-glucose hydrolase in E.coli. In fact I have found that whole cells of wild-type or dra^{c} mutants of S.typhimurium (or wild-type E.coli) are unable to catabolise external dRib-5-P, although they are able to do so if treated with toluene. Therefore crypticity due to changes in permeability to the substrate cannot be as easily dismissed as in the case of UDP-glucose hydrolase. It may be argued that since dra^{c} mutants are constitutive for thymidine phosphorylase, then dRib-5-P must enter the cell in order to act as an inducer. (One may speculate that this is due to a vectorial action of phospho-deoxyribomutase.) It is then possible that it cannot leave the cell and be degraded due to a permeability change; this implies that deoxyriboaldolase cannot degrade dRib-5-P which is internal to the membrane. Alternatively, it is possible that deoxyribo-aldolase can normally degrade internal dRib-5-P but in a dra^{c} mutant it is altered such that it cannot now do so.

Crypticity is favoured over the "two-enzyme" hypothesis for dra^{c} mutants, for reasons discussed earlier. It may also be pointed out that this explanation cannot be ruled out for the group three and group four mutants of Hoffee (1968), described above. The only observation grossly inconsistent with the hypothesis is that group four mutants are able to utilise deoxyribose. Thus it is possible that these anomalous tlr mutants are very similar to the dra^{c}

mutants described here.

(2) The Cytotoxicity of Sugar Phosphates

The deoxynucleotide sensitivity of dra⁻ strains seems to be a result of the accumulation of dRib-5-P (this investigation). It is now a common observation that sugars are toxic to mutants which are unable to catabolise phosphorylated derivatives of the sugar. Thus all the following sugar phosphates are toxic when they accumulate in mutants in which their catabolism is blocked (see also Bock & Neidhardt, 1966): L-ribulose-5-phosphate (Englesberg et al, 1962), L-α-glycerol-1-phosphate (Bock & Neidhardt, 1966b), D-galactose-1-phosphate (Kurahashi & Wahba, 1958; Yarmolinsky et al, 1959), L-rhamnulose-1-phosphate (Barkulis, 1949; Englesberg & Baron, 1959), glucose-6-phosphate (Fraenkel, 1968) and 6-phosphogluconate (H.L. Kornberg, personal communication). In addition, there is evidence that the accumulation of 3'-phosphoadenosine-5'-phosphosulphate is deleterious in Salmonella typhimurium (Gillespie et al, 1968). It is possible that all these examples of sugar phosphate toxicity listed above have in common the same mechanism of toxicity, i.e. they all act at the same target site. Alternatively, each case may be due to the inhibition of a different enzyme. Both alternatives will be discussed.

Some of the examples given above have in common the observation that glucose overcomes the inhibition of growth. This is the case

for toxicity due to arabinose (Englesberg et al, 1962) and glycerol (Cozzarelli et al, 1965) and glucose will partially overcome the inhibition induced by galactose (Yarmolinsky et al, 1959). As pointed out by Cozzarelli et al (1965) this is unlikely to be due to catabolite repression of enzymes necesssary for uptake of the sugar since the effect occurs immediately, before any enzyme could be diluted out by further growth. Two possibilities remain (Cozzarelli et al, 1965): firstly, glucose is very rapidly metabolised and may give rise to intermediates which by-pass the block caused by the sugar phosphate. This is unlikely to be a general explanation in view of the fact that glucose does not overcome toxicity caused by dRib-5-P. Also glucose only partially overcomes inhibition by galactose-1-phosphate (gal-1-P) (Yarmolinsky et al, 1959) and in fact inhibition is observed in a medium with glucose as sole carbon source (Kurahashi & Wahba, 1958). Secondly, glucose may reduce the rate of uptake of the sugars concerned. Cozzarelli et al (1965) tested this hypothesis by adding glucose and glycerol together to a strain sensitive to glycerol and accumulating α-glycerol-1-P. A transient inhibition was observed, and it was concluded that this was not the explanation.

Six hypotheses which would account for sugar phosphate toxicity in general, have been proposed.

(1) The phosphorylated sugars act as a phosphate trap, thereby starving the cell of intracellular phosphate (Kurahashi & Wahba, 1958).

(2) During the formation of most sugar phosphates, ATP is involved and it has been suggested that there is a depletion of cellular ATP, rather than inorganic phosphate (Kurahashi & Wahba, 1958).

(3) A variation of the first hypothesis is that sugar phosphates interfere with phosphate uptake; this has been considered in the present investigation.

(4) The synthesis of RNA is inhibited (see Bock & Neidhardt, 1966a,b).

(5) The uptake of the available carbon source is inhibited.

(6) Energy metabolism is affected.

The first hypothesis is unlikely in view of the fact that α-glycerol-1-P which can enter cells via a specific transport system (Lin *et al*, 1962) is also toxic in strains deficient in both glycerol kinase and glycerol dehydrogenase (Cozzarelli *et al*, 1965). This also makes the second hypothesis unlikely, as does the very fact of deoxynucleoside sensitivity in *dra*$^-$ strains, since in both cases ATP is not involved. With respect to the second hypothesis, the pool size of ATP was directly measured in cells inhibited by an accumulation of fructose 1:6 diphosphate (Bock & Neidhardt, 1966b). A small drop in the ATP level was found, but hardly enough to account

for the rapidity and severity of the inhibition observed.

The third hypothesis has been tested in the current investigation on deoxynucleoside sensitivity, with negative results. This also argues against the first hypothesis.

The fourth hypothesis was considered of particular relevance to deoxynucleoside sensitivity in view of the fact that ribonucleosides reverse the inhibition (Alikhanian et al, 1966; Beacham et al, 1968a). However, this can be satisfactorily explained in other ways; namely by their inhibiting enzymes leading to the formation of dRib-5-P. Furthermore I have found that ribonucleosides do not overcome inhibition by galactose in a transferase negative strain (strain NZ). The fact that RNA synthesis is severely inhibited within 6 minutes of the addition of thymidine is nevertheless consistent with this hypothesis.

Also consistent with this hypothesis are the results of Bock and Neidhardt. They found that a mutant which accumulates fructose 1:6 diphosphate shows strong inhibition of total protein, DNA and RNA synthesis in a complex medium containing amino acids, nucleotides and vitamins indicating that the sensitive site is most likely to be in one or more of the polymerisation reactions (Bock & Neidhardt, 1966a,b). Furthermore, the strain used has 'relaxed' amino acid control over RNA synthesis. This means that it would be unlikely for an inhibition of protein synthesis alone to lead

to a cessation of RNA synthesis. Bock and Neidhardt (1966b) suggest that the intracellular accumulation of sugar phosphates may, in this case, directly affect the synthesis of RNA.

The fifth hypothesis is that an intracellular pile-up of sugar phosphates feed-back inhibits the uptake of sugars used as a carbon source. For this to be true, the uptake of an extremely wide variety of carbohydrates and other carbon sources would have to be affected. Thus deoxynucleoside sensitivity is manifest on glucose, glycerol, ribose, pyruvate, gluconate, xylose, mannose, succinate and casamino acids as carbon sources and in nutrient and tryptone broth. L-arabinose sensitivity is observed with the following carbon sources: casein hydrolysate, fructose, mannose, xylose, arabinose, rhamnose, sorbitol, dulcitol, melibiose, glycerol and DL-alanine (Englesberg, et al, 1962). Mutants defective in the phosphotransferase system of sugar uptake are very pleiotropic (Simoni et al, 1964; Wang & Morse, 1968), but the mutants reported thus far do not cover the range of carbon sources listed above. The observation of sensitivity on complex media in the case of toxicity due to fructose 1:6 diphosphate, L-rhamnulose-1-P and L-ribulose-5-P also argues against this hypothesis. In the case of fructose 1:6 diphosphate sensitivity, the uptake of glycerol is totally abolished. This can be explained by an inhibition of

glycerol kinase activity by fructose 1:6 diphosphate (Zwaig & Lin, 1966). The uptake of glucose, the inhibitory sugar, is also 40% of normal. Inhibition by glucose is also seen using succinate as carbon source, showing that inhibition of glycerol uptake is not the cause of growth inhibition.

The last hypothesis is proposed because of the observation that respiration is rapidly reduced in the case of deoxynucleoside sensitivity (Fig. 11; see also Lomax & Greenberg, 1968). However, in the case of dRib-5-P toxicity (Table 1), sensitivity is still observed when the cells are grown anaerobically. Furthermore, no inhibition of the oxidation of glucose or peptone was observed in the case of toxicity due to L-rhamnulose-1-P (Barkulis, 1949).

In conclusion the most reasonable hypotheses are that sugar phosphates inhibit either RNA synthesis, or some aspect of energy metabolism. It is difficult to be more specific, partly due to a lack of data. It has been suggested, on the basis of experiments with yeast using uncoupling agents, that the energy of oxidative phosphorylation may be directly coupled to protein and/or RNA synthesis. That is, ATP itself is not involved. Alternatively, ATP from oxidative phosphorylation or glycolysis, may give rise, via specific enzymes, to high energy intermediates which may then be used for macromolecular synthesis (Jarett & Hendler, 1967). Kovac and Kuzela (1966) in similar experiments with E.coli conclude

that their data support the notion that glycolytically formed ATP is in equilibrium with high energy intermediates of oxidative phosphorylation which are used directly for protein synthesis (Slater, 1953). The high energy intermediates could also be necessary in a less direct way (Kovac & Kuzela, 1966).

If this kind of energy coupling does occur, it is conceivable that sugar phosphates may interfere with it at some stage. The data summarised above, are generally consistent with this hypothesis, although it is difficult to predict the consequences of such an inhibition, apart from an inhibition of RNA and protein synthesis. Thus on this hypothesis sugar phosphate toxicity is due to a block in energy utilisation.

None of the six hypotheses summarised above are mutually exclusive and each example of sugar phosphate toxicity may have a different cause.

The alternative type of explanation for sugar phosphate toxicity is that in each case a particular enzyme is inhibited which is essential for normal growth. Thus Kurahashi and Wahba (1958) suggest, on the basis of results by others, that galactose-1-P may inhibit phosphoglucomutase. Fructose 1:6 diphosphate inhibits glycerol kinase (Zwaig & Lin, 1966) but this is not an essential enzyme, except for growth on glycerol. Fructose 1:6 diphosphate sensitivity is also seen using succinate as carbon source (Bock &

Neidhardt, 1966b). The most convincing explanation of this type comes from the work of Fraenkel on glucose-6-P toxicity (Fraenkel, 1968). In this case sensitivity to glucose was manifest on a variety of carbon sources (glycerol, α-glycerol-P, fructose, succinate) but not on mannitol, xylose, or gluconate. This suggests that fructose 1:6 diphosphatase is inhibited since on the former carbon sources this enzyme is essential for the formation of hexose monophosphates, but this is not so for the latter carbon sources. Inhibition of fructose 1:6 diphosphatase by glucose-6-P was shown *in vitro*. It may be noted here that in the case of galactose-1-P and dRib-5-P toxicity, inhibition is still observed on xylose and mannitol (present studies) and L-ribulose-5-P inhibition is observed on xylose (Englesberg *et al*, 1962).

It has been suggested by Cozzarelli *et al* (1965) that many enzymes may have an affinity for a phosphate moiety, accounting for the fact that phosphorylated compounds are more often toxic than non-phosphorylated ones.

The two alternative types of explanation for sugar phosphate toxicity are not mutually exclusive. Thus in some cases a specific enzyme may be the sensitive target while in others a more general explanation might be true, or both.

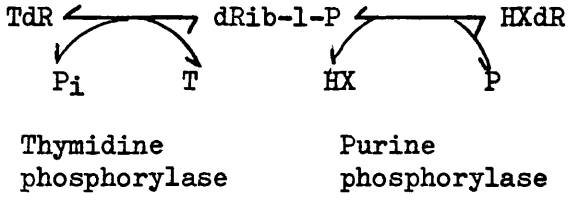
Finally, it may be noted that this phenomenon is of interest since it provides a model system for hereditary galactosemia.

This human "molecular disease", like galactose sensitivity in E.coli, is caused by the absence of galactose-1-P transferase and presumably the resulting accumulation of galactose-1-P.

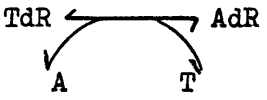
(3) Pathways for the Uptake of Exogenous Thymine

Two general mechanisms for the uptake of thymine from the medium for incorporation into DNA are possible: firstly via deoxyribosyl transferase (Boyce & Setlow, 1962; Munch-Petersen, 1967) and secondly, via thymidine phosphorylase (Kammen, 1967b; Beacham et al, 1968a). In the first case the deoxyribosyl donor would be a deoxynucleoside and in the second case dRib-1-P, in both cases the result being the formation of thymidine. Both mechanisms are consistent with the observation that deoxynucleosides potentiate thymine uptake in wild-type strains (Boyce & Setlow, 1962; Munch-Petersen, 1967; Kammen, 1967b) and that tlr mutants are dra$^-$ or drm$^-$ (see p.30 et seq.). Thus deoxynucleosides might either be involved in transdeoxyribosylation or they might give rise to dRib-1-P, a substrate for thymidine phosphorylase. Both deoxynucleosides and dRib-1-P might be expected to accumulate in dra$^-$ and drm$^-$ strains. First of all, the status of deoxyribosyl transferases will be briefly reviewed. It was shown in 1952 by Lampen that deoxyribosyl transferases specific for purines or pyrimidines existed in E.coli, but that transfer of the deoxyribosyl moiety between purines and pyrimidines required inorganic phosphate

(Lampen, 1952). This has been confirmed by Imada and Igarasi (1967) who also showed that purified purine and thymidine phosphorylases were together able to catalyse the transfer of deoxyribose from thymidine to hypoxanthine (HX). This suggested that purine-pyrimidine transfer requires the action of both phosphorylases and occurs via the formation of dRib-1-P, viz:



Munch-Petersen has assayed thymidine-adenine deoxyribosyl transferase in E.coli but this situation is slightly complicated by the use of extracts which were not dialysed and which might therefore contain residual phosphate (Munch-Petersen, 1967). Recently Munch-Petersen (1968b) found that adenine stimulated the cleavage of TdR to thymine by washed cells of E.coli, in the absence of added phosphate, and attributed this to transdeoxyribosylation since AdR was found in the reaction mixture, viz:



The effect of adenine was abolished when a pup$^-$ mutant was used, and also when the cells are sonicated. Arsenate enhanced the formation of AdR, while it decreased the phosphorolysis of TdR (by whole cells).

It was suggested that purine phosphorylase and thymidine phosphorylase, together with other enzymes of nucleoside catabolism, form a structural complex at the surface of the cell. The two phosphorylases by virtue of their proximity may then act together in a transdeoxyribosylation reaction without the intermediate formation of dRib-1-P.

These results do not conflict with those of Lampen (1952) or Imada and Igarasi (1967) for two reasons: (a) in neither case was adenine used. Munch-Petersen finds that guanine and hypoxanthine are inactive in her transdeoxyribosylation assay (Table 2 of Munch-Petersen, 1968b). (b) The former authors used cell free extracts whereas Munch-Petersen used whole cells.

An alternative to her conclusion, however, is that the activity observed is associated with the purine phosphorylase protein. That is, it is a bifunctional enzyme with phosphorylase activity being its major activity. (Very recently evidence has appeared indicating that thymidine phosphorylase has purine-pyrimidine transferase activity; thus the latteractivity can be assayed in dialysed extracts, but it is lost concurrently with the loss of thymidine phosphorylase (Mineeva & Il'ina, 1969)). The only observation unexplained by this hypothesis is the decrease in activity upon sonication. However, this also occurs with the spontaneous cleavage of thymidine in the absence of phosphate

and adenine, and is also unexplained. Furthermore, in the absence of data on the effect of phosphate on thymidine-adenine deoxyribosyl transfer, in sonic extracts, the intermediate formation of dRib-1-P cannot be ruled out since the extracts were not dialysed. Phosphate does not stimulate the reaction with washed cells, but neither does it with thymidine alone.

The following tentative conclusions concerning purine-pyrimidine deoxyribosyl transfer may be made:

(1) Purine-pyrimidine deoxyribosyl transfer activity between deoxyguanosine and hypoxanthine (and vice versa) and between thymidine and hypoxanthine is due to the intermediate formation of dRib-1-P, i.e. the coupling of two phosphorylases, (Lampen, 1952; Imada & Igarasi, 1967).

(2) Thymidine-adenine deoxyribosyl transferase activity either has a similar basis or is due to two activities of one enzyme, or due to the proximity of two phosphorylases at the cell surface.

(3) Deoxyadenosine and deoxyguanosine-thymine deoxyribosyl transfer is associated with thymidine phosphorylase (Mineeva & Il'ina, 1969) (An unspecified purine-pyrimidine deoxyribosyl transferase has also been reported to be associated with E.coli thymidine phosphorylase (Vilstrup, quoted in Munch-Petersen, 1968b))

In all these cases except (3), purine nucleoside phosphorylase is required for purine-pyrimidine deoxyribosyl transfer, and in the

last instance thymidine phosphorylase is required. There is no evidence for a distinct purine-pyrimidine deoxyribosyl transferase.

Purine specific and pyrimidine specific deoxyribosyl transferase activity has been reported in E.coli and it has been stated that these activities can be separated from the phosphorylases by fractionation of the extracts (Lampen, 1952). In addition pyrimidine specific transferase activity has been reported to be associated with purified E.coli thymidine phosphorylase (Zimmermann, 1964).

The deoxyadenosine-potentiated incorporation of external thymine (8 μM) in wild type E.coli requires the presence of thymidine phosphorylase (Budman & Pardee, 1967). Tlr mutants of E.coli which lack thymidine phosphorylase are unable to grow on low (20 μM) or very high (1 mM) concentrations of thymine (Table 12). Novick (quoted in Denhardt, 1969) also finds that tpp$^-$ mutants are unable to grow on thymine. These observations lead to the conclusion that thymidine phosphorylase is the main route for the uptake of exogenous thymine.

Purine-pyrimidine deoxyribosyl transferase was postulated to be involved in AdR mediated uptake in wild type cells (Munch-Petersen, 1967). From the considerations above, it may be predicted that AdR potentiated thymine uptake should not occur in a pup$^-$ strain, since it seems that purine phosphorylase is necessary for observed purine-pyrimidine deoxyribosyl transferase. This, in fact, is the case (Munch-Petersen, 1968b).

The ability of *pup*$^+$ *tpp*$^-$ strains to grow with high concentrations (1 mM) of thymine (but not 20 μM thymine) plus AdR or GdR is also considered to be due to purine-pyrimidine deoxyribosyl transferase activity. This is not inconsistent with the result of Budman and Pardee (1967) mentioned above, as they used low (8 μM) thymine plus AdR. If this is the case then, since thymidine phosphorylase is not required, some purine-pyrimidine transferase activity must be associated with purine phosphorylase. A *pup*$^-$ *tpp*$^-$ mutant is unable to grow with thymine plus AdR.

Such a strain is able to grow, however, with CdR or UdR plus thymine; this observation is consistent with the existence of a discrete pyrimidine specific deoxyribosyl transfer (Lampen, 1952).

In summary, there are three possible mechanisms for the conversion of thymine to thymidine and the following conclusions are made:

(1) Thymidine phosphorylase: This is undoubtedly the main route for the incorporation of thymine by a *thy*$^-$ (*tlr*$^+$ or *tlr*$^-$) mutant growing on thymine. It is also involved in the AdR potentiated incorporation of low concentrations of external thymine in *thy*$^+$ strain of *E.coli*.

(2) Purine-pyrimidine specific deoxyribosyl transferase: Such an enzyme almost certainly does not exist in *E.coli*, but this activity

may be associated with purine phosphorylase, thymidine phosphorylase, or result from the concerted action of both enzymes. Such activity does play a part in thymine uptake if purine deoxynucleosides and thymine are present.

(3) Pyrimidine specific deoxyribosyl transferase: This enzyme may function in thymine uptake if pyrimidine deoxynucleosides and high concentrations of thymine are present.

Thus, with the use of a suitable deoxyribosyl donor, thymine is converted to thymidine. A thymidine kinase is then available for the conversion of TdR to TMP (Okazaki & Kornberg, 1964) which is on the normal metabolic pathway to DNA (see Chart 1 ~~Fig. 18~~).

(4) The Surface Localisation of Enzymes of Deoxynucleoside Catabolism

It was first observed by Malamy and Horecker (1964) that the alkaline phosphatase of E.coli is released into the medium when cells are converted into spheroplasts by treatment with EDTA and lysozyme. Other enzymes have subsequently also been found to be released by this treatment, viz: 5'-nucleotidase, acid phosphatase, cyclic phosphodiesterase (Neu & Heppel, 1964; 1965), DNAase I (Cordonnier and Bernardi, 1965) and UDPGase and ADPGase (Melo & Glaser, 1966). All these enzymes are also selectively released by osmotic shock, a treatment which involves exposure of the cells to 20% sucrose and 1 mM EDTA in Tris-HCl buffer followed by rapid

dispersal in ice-cold water (Neu & Heppel, 1965; Nossal & Heppel, 1966). Both of the above procedures do not release many other control enzymes. It was suggested by Malamy and Horecker (1964) that alkaline phosphatase is localised in the periplasmic space, a region between the cell membrane and the cell wall (Mitchell, 1961). Evidence for the surface localisation of alkaline phosphatase and 5'-nucleotidase, external to the cell membrane, has been obtained using histochemical techniques (Done *et al*, 1965; Spicer *et al*, 1966; Nisonson *et al*, 1969). Furthermore, alkaline phosphatase is selectively released into the medium during growth of a mutant of *E.coli* which has a defective wall and is osmotically sensitive (Mangiarotti *et al*, 1966). Evidence of a different kind is available in the case of alkaline phosphatase, cyclic phosphodiesterase, and 5'-nucleotidase. It is based on the premise that phosphorylated compounds, with the exception of a few, will not pass freely through the cell membrane. In the case of nucleotides this has been substantiated by Lichtenstein *et al* (1960) who found that the phosphate moiety of dCMP is excluded from the cell, although the cytosine moeity was utilised. It has been observed that cyclic UMP and AMP, substrates for cyclic phosphodiesterase and 5'-nucleotidase respectively are hydrolysed by

intact cells; the same is true of various substrates of alkaline phosphatase (Brockman & Heppel, 1968; Neu, 1967b). This is taken to indicate that these enzymes are localised exterior to the cell membrane.

In the case of enzymesof deoxynucleoside catabolism the evidence for surface localisation is restricted to their release by osmotic shock (Kammen, 1967b; Munch-Petersen, 1967; 1968b). The surface localisation of these enzymes poses several interesting questions.

(1) Are they able to metabolise internal or external substrates, or both?

(2) Are the substrates of surface enzymes able to penetrate the cell?

(3) Why are they surface localised?

Needless to say, it is difficult to devise experimental approaches to these problems. However, a clue to the first and second questions derives from the fact that one of the intermediates in deoxynucleoside catabolism, dRib-5-P, is an inducer. As such it seems clearly necessary that it should neutralise 'repressor', or otherwise interact with an operator locus, in order to initiate translation. For this to occur, it would seem necessary that dRib-5-P should exist inside the cell; this being the case it must

somehow be metabolised in order to avoid a vast intracellular accumulation. Deoxyriboaldolase and phosphodeoxyribomutase, and (unspecified) phosphatases are the only known enzyme for the dissimilation of dRib-5-P, and all are surface enzymes (Brockman & Heppel, 1968; Dvorak et al, 1967; Munch-Petersen, 1968b). It therefore seems likely that internal dRib-5-P is metabolised via surface enzymes. One alternative, however, is that dRib-5-P is excreted. This is less likely in view of the fact that excreted deoxyribose is always found in the free form (Breitman & Bradford, 1964).

Another alternative is that the membrane is permeable to dRib-5-P; the latter would therefore be accessible to an enzyme in the periplasmic space. This also seems unlikely in view of the fact that, as already mentioned, whole cells of both E.coli and S.typhimurium are unable to degrade dRib-5-P.

There is in fact no evidence that any of the enzymes of deoxynucleoside catabolism are able to hydrolyse an external substrate. Thus the possibility remains that, although the enzymes are probably located at the cell surface and all the reactions may take place at the cell surface, the sequence of reactions takes place inside the cell membrane.

With respect to the third question, two alternative are possible. Firstly, enzymes may be surface localised in order to

protect internal substrates from degradation. In view of the considerations above, this seems unlikely in the case of enzymes of deoxynucleoside catabolism. Secondly, an enzyme may be surface localised in order to hydrolyse compounds in the external medium. In view of the possible 'scavenging' role of surface enzymes (see later, p.1)2 et seq.) this possibility is favoured, at least in the case of purine and pyrimidine phosphorylases. However, it is not clear why deoxyribomutase and deoxyriboaldolase are also surface localised.

In the case of phosphodeoxyribomutase, it is possible that dRib-1-P is produced in the periplasm and is impermeable to the cell. The enzyme would therefore have to be surface localised in order to metabolise it. If dRib-5-P were also impermeable, deoxyriboaldolase would also have to be surface localised. However, if uridine phosphorylase produces dRib-1-P then, since the latter is an intracellular enzyme (Heppel, 1967), dRib-1-P would not be produced outside the cell. Secondly, as discussed above, since dRib-5-P is an inducer, it seems likely that it penetrates the cell. It may be noted that this does not imply that the cell membrane is freely permeable to dRib-5-P since it is conceivable that phosphodeoxyribomutase is capable of acting vectorially, i.e. its product, dRib-5-P is produced inside the cell.

Perhaps a more attractive hypothesis for the surface location

of these two enzymes is that it is a result of their belonging to a single unit of transcription and translation, together with purine phosphorylase and thymidine phosphorylase. This hypothesis makes the interesting prediction that extreme polar mutations anywhere in either of the four genes specifying these four enzymes should result in the loss of all four enzymes, rather than just the enzymes specified by the distal genes. However, since almost nothing is yet known about the synthesis of surface enzymes in bacteria, this will not be discussed further.

In summary, it is proposed that purine and thymidine phosphorylases are surface located in order to hydrolyse external substrates. Deoxyribomutase and deoxyriboaldolase are similarly located possibly because they belong to the same unit of transcription and translation.

Finally, it may be pointed out that if uridine phosphorylase is involved in the production of dRib-1-P (see Chapter III), then it may be significant that this enzyme is intracellularly located (Heppel, 1967), in contrast to purine phosphorylase and thymidine phosphorylase. Thus if deoxynucleosides arise intracellularly (from deoxynucleotides), then they may be degraded by uridine phosphorylase before they reach the surface of the cell where the other nucleoside phosphorylases are located. That is, internally produced deoxynucleosides may be more accessible to uridine phosphorylase than purine or thymidine phosphorylases, and the former enzyme may

therefore be almost solely concerned with the production of dRib-1-P.

CHAPTER V

Deoxyribomutase-Negative Strains of E.coli

It has been argued previously that thymidine is not toxic to *drm*$^-$ strains of *E.coli* because the rate of breakdown to dRib-1-P, relative to its rate of synthesis, is not high enough, i.e. the rate of excretion of dRib-1-P, as deoxyribose, is too high to allow a sufficient accumulation of dRib-1-P to inhibit growth.

An attempt was therefore made to isolate mutants constitutive for thymidine phosphorylase in a *drm*$^-$ strain in order to determine whether TdR would now prove toxic to such a strain. It was assumed that enzymes of deoxynucleoside catabolism constitute an operon; a strain constitutive for purine phosphorylase should therefore also be constitutive for thymidine phosphorylase.

Purine nucleoside phosphorylase is non-specific with respect to deoxy- and ribonucleosides (Ahmad *et al*, 1968) and so it was considered that a strain constitutive for this enzyme would grow faster with guanosine as sole carbon source than a *dra*$^+$ strain. To see if this was the case, strains C600, CR34 and P152 were streaked on minimal agar plates containing 2.5-5 mM guanosine as sole carbon source. CR34 (*dra*$^-$) did appear to behave differently from P152 and C600 in that after 20 hr incubation growth was seen and the streak had an abnormally white appearance. This is assumed

to be due to a precipitate of guanine. C600 showed less growth and a less marked precipitate but surprisingly, P152 showed neither growth nor a precipitate. That P152 grows very little can be explained by the finding that mutase is also non-specific with respect to ribo- and deoxyribosides (Kammen, personal communication). Nevertheless, one might expect a little growth, due to phosphatase action on Rib-1-P followed by conversion of the resulting free ribose to Rib-5-P. However, no growth at all is observed even after four days incubation.

However, mutants able to grow on guanosine as sole carbon source were obtained. It was attempted to determine (a) why drm$^-$ strains show a total inability to grow on nucleosides and (b) the nature of the mutants now able to do so.

Selection of Mutants

Mutants were selected from P152 on plates containing 5 mM guanosine as carbon source, and 400 µM thymine. The latter was included to minimise any possible inhibitory effect of guanosine on the growth of thymine requiring strains. (It is known that uridine inhibits growth of thy$^-$ mutants: this is presumably due to trapping of thymine as ribosyl thymine (Mantsavinos & Zamenhof, 1961; Denhardt, 1969) and inhibition of thymidine phosphorylase (Table 20; see also Budman & Pardee, 1967)). However no inhibition

of growth of P152 was seen on plates containing 5 mM guanosine and 400 µM thymine and also 0.4% glucose or 20 mM glycerol as a carbon source. Colonies were picked and purified after about 36 hr of growth. When spontaneous mutants were selected, only one was obtained from approximately 5 x 10^8 cells plated, but many more were obtained when cells were mutagenised with NMG.

General Properties of Mutants

P152R1, the spontaneous mutant, was still *tlr*$^-$ and had no detectable phosphodeoxyribomutase but it had now become thymidine sensitive and inducible (by thymidine) for thymidine phosphorylase. It was unable to grow on thymidine as carbon source. All the other mutants (152-1, 152-2, 152-6, 152-7, 152-8) were *tlr*$^+$, not thymidine sensitive, and were able to grow on thymidine as a carbon source. They were probably *drm*$^+$. P152R1 had the further interesting property of being constitutive for enzymes of deoxynucleoside catabolism when grown on glycerol (or succinate) as carbon source, but not on glucose (Table 26). This can be explained by very strong catabolite repression and will be discussed later. Strains 152-1 and 152-8 had also become constitutive but on both glucose and glycerol as sole carbon sources. Strain 152-7, 152-2 and 152-6 were not constitutive.

Growth of P152 and P152-R1 on Ribonucleosides as Sole Carbon Source

In investigating the failure of P152 to grow on nucleosides, strain P152-R1 was included for comparison; P152-R1 has no deoxyribo-

TABLE 26. EFFECT OF CARBON SOURCE ON ENZYME LEVELS IN P152-R1 AND ITS DERIVATIVES

Strain	Deoxyriboaldolase		Thymidine phosphorylase		Purine phosphorylase	
	Glucose	Glycerol	Glucose	Glycerol	Glucose	Glycerol
CR34	0	0	1954	1063	379	324
P152	16	17	124	82	105	173
P152-R1	15.5	113.5	132	912	90	269
P152-R1 *drm*$^+$	24.5	102	152	808	116	219
P152-R1 *thy*$^+$			111	423		

Enzyme was assayed in sonic extracts in all cases. Strain CR34 was included as a control.

mutase activity, and it was hoped to determine the basis of its ability to grow on nucleosides. Figure 18 shows the excretion of free ribose from uridine in strains P152 and P152-R1 in minimal medium without thymine. The latter was omitted to avoid trapping of Rib-1-P to form ribosyl thymine. It can be seen that P152 does excrete ribose at a low rate which is less than one tenth that of P152-R1. The ribose excreted by P152-R1 is also taken up again.

The ribose excreted is not in the form of Rib-1-P, as shown by the fact that not more than 10% of the ribose is precipitable with $ZnSO_4$ and $Ba(OH)_2$. No excretion is observed in either strain without uridine.

The high rate of excretion in P152-R1 could be due either to increased levels of uridine phosphorylase, or to increased phosphatase activity on Rib-1-P. Table 27 shows that a sonic extract of P152-R1 is able to hydrolyse Rib-1-P, whereas P152 is unable to do so. It might be noted, however, that sonic extracts of P152 do sometimes show a background appearance of free ribose with and without added Rib-1-P, amounting to about 10% of that of P152-R1 with Rib-1-P. Table 27 also shows that P152-R1 has a three-fold higher level of uridine phosphorylase. The low rate of appearance of ribose from uridine in P152 presents an apparent discrepancy with the fact that no Rib-1-P phosphatase can be detected, but this may be due to nucleoside hydrolase activity.

Fig. 18. Excretion of Ribose from Uridine in Strains P152 and P152-R1

Cultures were grown in M9 medium containing glycerol as a carbon source and 20 μM thymine to an OD_{600} of 0.12. These were then filtered, washed and resuspended in the same medium without thymine and with 200 μM uridine. Samples were taken at intervals, filtered through a millipore filter and ribose measured by the orcinol reaction. (●, P152-R1; +, P152)

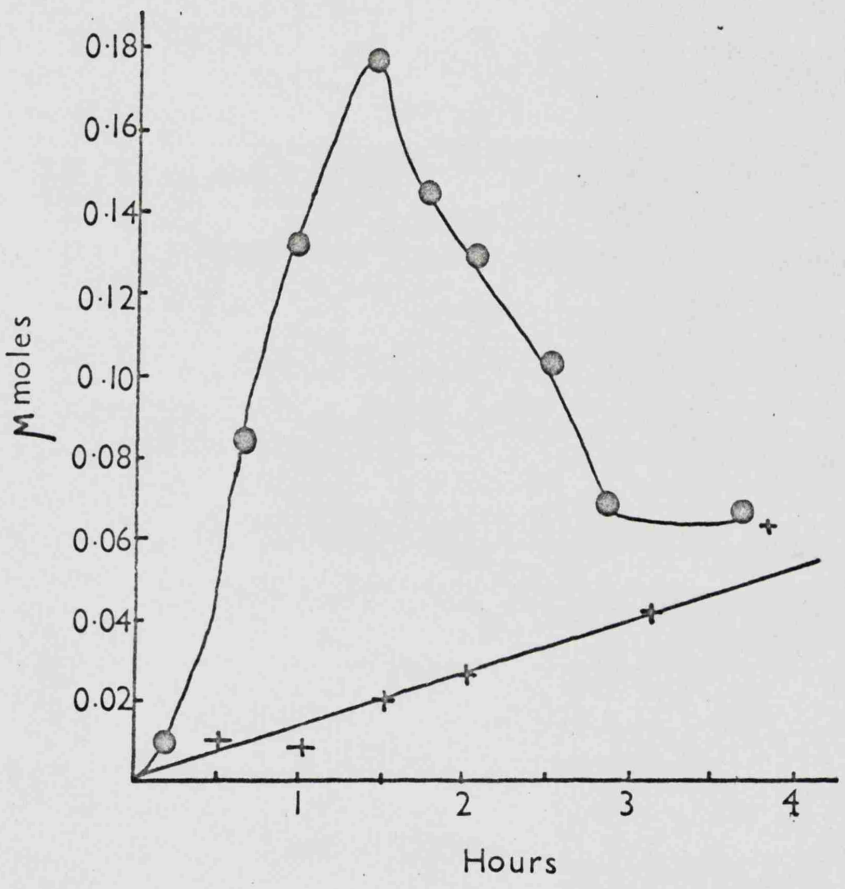

0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
M moles
1
2
3
4
Hours

TABLE 27. URIDINE PHOSPHORYLASE AND PHOSPHATASE ACTIVITY IN P152-R1

Strain	Uridine phosphorylase	Phosphatase	
		Substrate: Rib-1-P	dRib-1-P[b]
152-R1	330	10.4	0.11
152	131	n.d.[a]	0.27
154	110	n.d.	0.39

[a] n.d. = not detectable.

[b] This level of activity is difficult to determine with accuracy.

The conversion of Rib-1-P or dRib-1-P to the free sugar was determined in cell suspensions treated with toluene. The reaction mixture contained 0.9 ml of toluenised cells and 0.1 ml of 20 mM Rib-1-P or dRib-1-P. Samples (0.05 ml) were taken about every 15 min into chilled water, and Rib-1-P or dRib-1-P, precipitated by the addition of $ZnSO_4$ (0.3 M; 0.1 ml) and $Ba(OH)_2$ (0.15 M; 0.2 ml). Ribose and deoxyribose was determined in the supernatant as described in Methods. Uridine phosphorylase was assayed in sonic extracts.

The phosphatase in P152-R1 is inhibited by P_i (20 mM) but not by sodium fluoride (50 mM) or 8-hydroxyquinoline (1 mM). The latter observation indicates that the enzyme is unlikely to be alkaline phosphatase since this is a Zn-metallo enzyme and is strongly inhibited by 8-hydroxy-quinoline (Garen & Levinthal, 1960). No activity is observed at pH 5.5 and only a 50% rise in activity is observed when the enzyme is measured at pH 8 rather than pH 7. The enzyme does not seem to hydrolyse dRib-1-P since sonic extracts of both P152 and P152-R1 hydrolyse this compound at the same low rate (Table 27). Thus P152 has undetectable Rib-1-P phosphatase activity whereas a mutant of this strain, P152-R1, which has regained the ability to grow on ribonucleosides, has high Rib-1-P phosphatase activity. It can therefore be reasonably argued that the complete failure of P152 to grow on ribosides is due to the fact that the rate of production of ribose from uridine is too slow. That is, the rate of supply of energy from uridine is too slow to support net growth. Ribose requires a permease for uptake (Eggleston & Krebs, 1959) and this would tend to exaggerate any energy deficiency.

The Nature of Strain P152-R1

Besides the appearance of a phosphatase able to hydrolyse Rib-1-P, P152-R1 is inducible for thymidine phosphorylase and sensitive to thymidine. These latter two properties suggest that

dRib-5-P can be formed in this mutant; the thymidine sensitivity further suggests that the dRib-5-P cannot be degraded. This being the case, the constitutivity of P152-R1 could be due to endogenous induction by dRib-5-P. If this is so then this constitutivity should depend upon P152-R1 being *thy*$^-$ (see Chapter III). Accordingly, *thy*$^+$ transductants of P152-R1 were made and enzyme levels determined. However, it can be seen from Table 26 that P152-R1 *thy*$^+$ is still constitutive. Furthermore a *drm*$^+$ revertant of P152-R1 (isolated on the basis of growth on thymidine as a sole carbon source) is also still constitutive (Table 26). Assuming that the genes specifying enzymes of deoxynucleoside catabolism constitute an operon, it is considered more likely that P152-R1 is constitutive due to a mutation in a regulator gene than to endogenous induction.

The reason why P152-R1 is sensitive to thymidine and inducible by thymidine is unclear. It can only be suggested that dRib-1-P accumulates in this strain sufficiently to be toxic (since it is inducible, although *drm*$^-$). The apparent inducibility of thymidine phosphorylase may be a consequence of a pile-up of dRib-1-P, perhaps by antagonising catabolite repression.

Although the nature of strain P152-R1 is unclear, the appearance of an alternate pathway for the catabolism of Rib-1-P is very striking; it is an example of what has been termed "indirect suppression" (Gorini & Beckwith, 1966). This term has been used to

define the situation where a mutation is suppressed by a mutation, usually in another gene, "which acts indirectly to circumvent the primary genetic lesion". It is distinct from intragenic and informational suppression in which the function of the original mutated gene is restored.

An example of indirect suppression particularly pertinent to the present discussion is the appearance of a phosphatase in yeast (Heredia & Sols, 1964). In this case a mutant was isolated which was resistant to the toxic effect of 2-deoxyglucose and was found to possess a phosphatase capable of hydrolysing deoxyglucose-6-phosphate, thereby preventing its intracellular accumulation.

Catabolite Repression

As discussed above, P152-R1 is constitutive on glycerol but not on glucose as a carbon source. This can be interpreted in terms of very strong catabolite repression by glucose. Thus in its presence, the synthesis of enzymes of deoxynucleoside catabolism are repressed to wild-type levels, while in its absence high levels are observed. This does not seem to be a general effect since normal catabolite repression of β-galactosidase is observed; i.e. the differential rate of induced enzyme synthesis is about two-fold less on glycerol than on glucose as a carbon source (see also Brown in Discussion in Magasanik, 1961; Moses & Yudkin, 1968) in both P152 and P152R1.

Catabolite repression sensitive mutants have been observed in at least three other systems: (1) the D-serine deaminase system (McFall, 1964). In this case it has been concluded that the extra catabolite repression is the result of a pleiotropic mutation in a regulator gene of the i-type (McFall, 1967). (2) The leucine operon (Friedman & Margolin, 1969). This is a biosynthetic group of enzymes and are not normally subject to catabolite repression. Evidence is presented that the mutation is in the operator gene. It is proposed that the operator has an altered specificity such that it now binds a 'foreign' repressor involved in catabolite repression of other enzymes. (3) Isocitrate lyase (Kornberg, 1964); constitutive mutants are sensitive to catabolite repression.

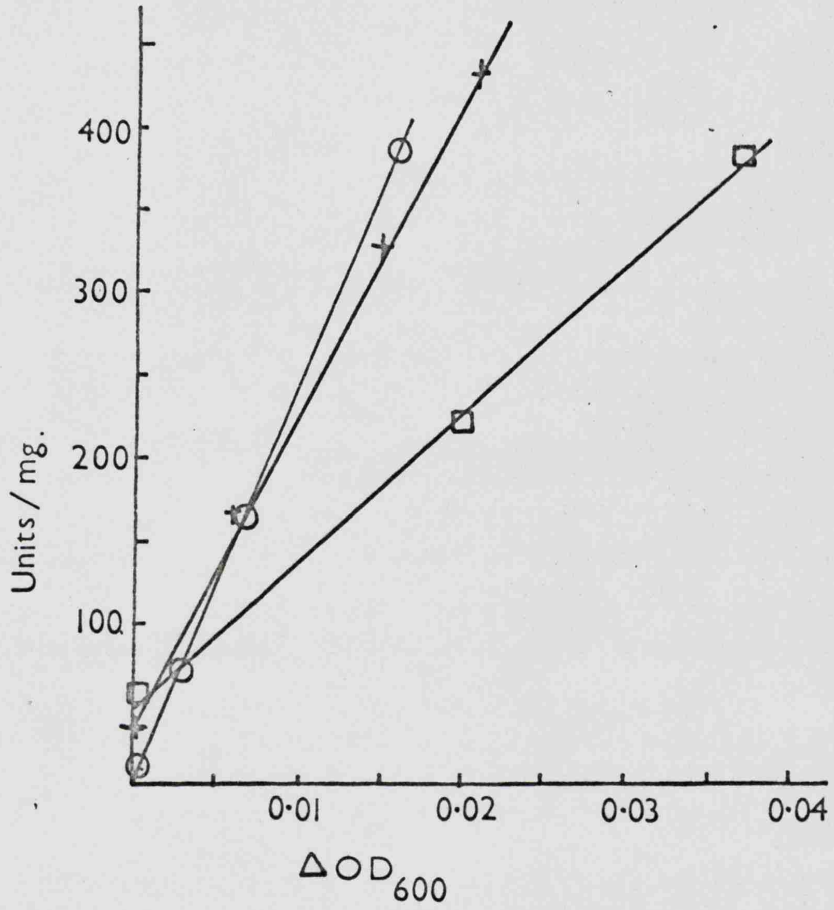
If one takes the view that P152-R1 carries a regulator mutation, then it is perhaps reasonable to suppose that this mutation is pleiotropic and evokes strong catabolite repression.

This raises the interesting and important question of whether or not thymidine phosphorylase is subject to catabolite repression in wild-type strains of E.coli.

Catabolite repression is characterised by a repression of enzyme synthesis by glucose as compared to other, less readily assimilated carbon sources (see Magasanik, 1961). Figure 19 shows that the differential rate of thymidine phosphorylase synthesis on glycerol or succinate is twice that on glucose as carbon source.

Fig. 19. Differential Rate of Thymidine Phosphorylase Synthesis in E.coli grown on Different Carbon Sources

Cultures were grown in exponential phase on the appropriate carbon source and TdR (2 mM) added at an OD_{600} of 0.12. Samples (50 ml) were taken during a period of about 23 min, immediately filtered through a millipore filter, washed and resuspended in 0.01 M Tris pH 7.2. The samples were then sonicated and assayed for thymidine phosphorylase. (□, glucose; +, glycerol; 0, succinate).


400
300
200
100
Units / mg.
0·01
0·02
0·03
0·04
ΔOD600

Mandelstam (1961) has shown that any carbon source, whether readily assimilated or not, when added to non-growing cells brings about a very strong repression of β-galactosidase. According to the theory of catabolite repression, anabolism is prevented thus allowing a large accumulation of catabolites from the carbon source used. Under these conditions even succinate which normally de-represses enzyme synthesis, with respect to glucose, strongly represses β-galactosidase. Figure 20 shows that the rate of synthesis of thymidine phosphorylase is reduced about six-fold when the cells are starved for threonine and leucine. These results, taken together, strongly indicate that thymidine phosphorylase, at least, is subject to catabolite repression.

Discussion

Strain P152-R1 is clearly pleiotropic. Thus this mutant has the following properties.

(1) It is inducible for thymidine phosphorylase by thymidine, but drm$^-$.

(2) It is sensitive to thymidine, although it is drm$^-$.

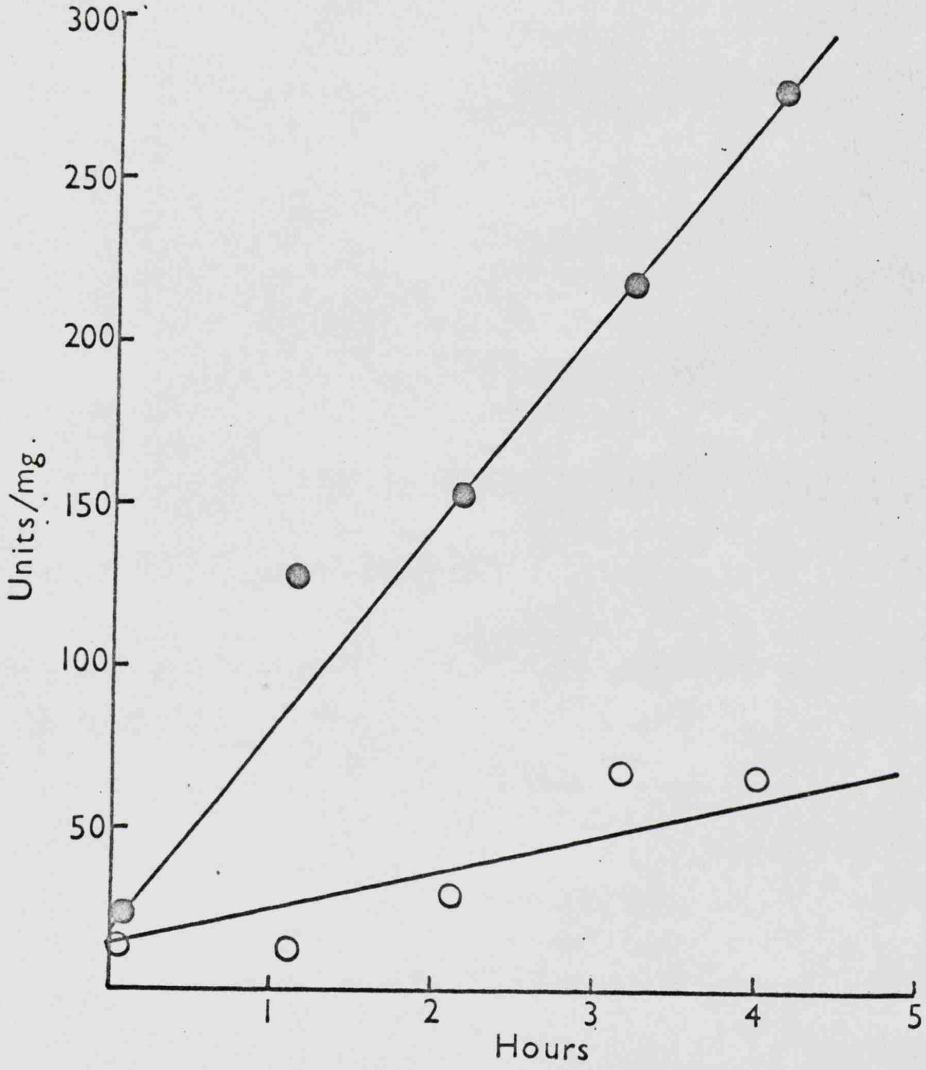
(3) It has acquired the ability to grow on nucleosides, while remaining drm$^-$. This property is attributed to the ability of this strain to hydrolyse Rib-1-P.

(4) It is constitutive for enzymes of deoxynucleoside catabolism but only when grown on carbon sources other than glucose.

(5) It has a higher uridine phosphorylase level than P152.

Fig. 20. Thymidine Phosphorylase Synthesis in Non-growing E.coli

An exponentially growing culture in succinate minimal medium was harvested, washed and resuspended in M9 salts (●) and M9 salts plus succinate (○). Both contained 2 mM TdR. Samples were taken and assayed as for Fig.19.


300
250
200
150
100
50
Units/mg.
1
2
3
4
5
Hours

It is difficult to account for all these properties on the basis of a single mutation. More likely, the strain is a double mutant. Thus a regulator mutation may confer constitutivity and enhanced catabolite repression (see above). A second mutation may be responsible for the observed Rib-1-P hydrolase activity. It may be speculated that the first two properties above, are a consequence of large amounts of intracellular dRib-1-P. Thus the apparent induction by TdR, may in fact be a result of an antagonism of catabolite repression due to the accumulation of dRib-1-P. TdR sensitivity in this strain may be a case of sugar phosphate toxicity due to dRib-1-P accumulation. However, neither of the two postulated mutations accounts for the increase in uridine phosphorylase.

Another problem (which also applies to the other constitutive revertants) is why strain P152-R1 should have become pleiotropic; only growth on guanosine was selected, which should only require the Rib-1-P hydrolase activity. It is difficult to understand what other selective pressures may have been operating.

CONCLUSIONS

(1) The Physiological Role of Enzymes of Deoxynucleoside Metabolism

In discussing this point it is important to establish whether these enzymes are normally involved in catabolism or anabolism. The evidence for the former seems overwhelming:

(1) Deoxyriboaldolase has a rather low affinity for acetaldehyde (Racker, 1952). The intracellular concentration of acetaldehyde is likely to be too low for efficient synthesis of dRib-5-P via deoxyriboaldolase. From studies on *tlr* mutants in this thesis and elsewhere, it can be seen that the intracellular concentration of dRib-1-P in wild type cells is also too low to allow synthesis of thymidine via thymidine phosphorylase.

(2) The enzymes concerned are inducible, a property of catabolic enzymes.

(3) They are all localised near the cell surface (Munch-Petersen, 1967; 1968b; Kammen, 1967a; This thesis). All the enzymes released by osmotic shock, so far found, are degradative.

(4) The pathway is subject to catabolite repression, a property of catabolic systems (Magasanik, 1961).

In addition there is evidence that the pathway is not normally used for the synthesis of deoxyribose (see Reichard, 1967).

(1) From isotope experiments (see Sable, 1966) using labelled small molecules (e.g. glucose, acetate), no evidence for *de novo* synthesis

of deoxyribose via deoxyriboaldolase was obtained.

(2) Purine and pyrimidines (except orotic acid) are not synthesised de novo, yet these are required substrates for both purine and thymidine phosphorylase (orotic acid is not a substrate for thymidine phosphorylase).

(3) The existence of mutants defective in one or more of the enzymes of this pathway, which have normal growth, is strong evidence that the enzymes are not normally required in biosynthesis.

One other aspect of the deoxynucleoside enzymes pertinent to this discussion, is the induction pattern. Although all the enzymes are induced by dRib-5-P, two of the enzymes, purine phosphorylase and phosphodeoxyribomutase, are also induced by the ribonucleosides adenosine and guanosine (Munch-Petersen, 1968b; Kammen, personal communication); these two enzymes are also non-specific for the pentose moiety, i.e. they degrade both ribo- and deoxyribonucleosides. Thus, whatever the function of this pathway, it would seem that both ribo- and deoxyribonucleosides should be considered.

Assuming that deoxyribonucleotides are dephosphorylated to give deoxyribonucleosides, it is possible to suggest four hypotheses as to the normal function of this pathway.

Firstly it is conceivable that these enzymes are part of a larger system concerned with the catabolism of DNA (Barth et al, 1968).. This DNA may originate from bacteriophages and serve as a

source of nucleotides via the restriction enzymes (Arber, 1968; Yuan & Meselsen, 1968). It may be significant that there is evidence that the restriction enzymes, as one may expect, are also surface localised (Glover & Schell, 1966). This hypothesis initially gained credence from the fact that the genes responsible for the restriction of foreign DNA (the hsp locus) are located in the same region of the linkage map of E.coli (see Taylor & Trotter, 1967) as the tlr locus, allowing the possibility that both loci may constitute a single gene cluster. However, the co-transduction frequency of hsp and tlr is only about 20% (Ahmad & Pritchard, 1969). There seems little reason why the deoxyribose of external DNA should be completely degraded, unless its use as a carbon source has proved of value in the metabolic economy of E.coli.

Secondly, the enzymes may be primarily concerned with the degradation of deoxynucleotides, rather than DNA. The production of deoxyribonucleotides via ribonucleotide reductase is subject to a complex system of allosteric control (see Reichard, 1967). One aspect of this control is that dATP completely inhibits the reductase, and thereby the synthesis of all four deoxynucleotides. Thus any situation leading to an excess of dATP over the other deoxynucleotides would be potentially lethal to the cell since the cell would be deprived of the other three deoxynucleotides required for DNA

synthesis. A similar situation applies to TTP which feedback inhibits (in the presence of ATP) the reduction of both CDP and UDP. An inducible pathway for the catabolism of excessive quantities of dATP and TTP would prevent such a lethal situation arising.

Thirdly, the pathway may exist in part, for the degradation of deoxyuridine nucleotides. An enzyme is present in E.coli for the conversion of dUTP to dUMP and it is thought this is to prevent the incorporation of dUTP into DNA (Bertani et al, 1963). It is conceivable that under certain conditions, for example when ribonucleotide reductase is de-repressed (Biswas et al, 1965), dUDP and dUTP may arise faster than they can be metabolised. An inducible pathway for their catabolism may then be an advantage.

These three hypotheses do not account for the fact that the group of enzymes concerned are involved in the catabolism of ribo-compounds as well as deoxyribo-compounds.

Finally, it has been suggested that nucleoside phosphorylases may serve to salvage the energy from nucleosides which escape from the cell (Friedkin & Kalckar, 1961). Perhaps it is more likely that this argument would apply to nucleotides and deoxynucleotides, since these are normally synthesised inside the cell. As Freidkin and Kalckar, (1961) have pointed out, the leakage of phosphorylated compounds from the cell is a sign of catastrophe; the utilisation of energy derived from these compounds might be important in the

repair and recovery of the cell. The surface location of the enzymes is consistent with this hypothesis. Furthermore, Kornberg (1962) has cogently argued that phosphorylases in general (in contrast to pyrophosphorylases) serve principally in degradative pathways to "conserve the energy of glycosidic and phosphodiester linked compounds". This hypothesis is considered the most likely, but an experimental test is obviously difficult.

(2) The Use of Labelled Thymine and Thymidine in Measuring DNA Synthesis

With wild type cells of E.coli, labelled TdR can be used for pulse labelling studies. After a short time however, breakdown of the TdR occurs due to thymidine phosphorylase. Labelling can be prolonged, by adding deoxynucleosides to the medium (Boyce & Setlow, 1962) or by using a tpp$^-$ mutant (Fangman & Novick, 1966).

The addition of deoxynucleosides to the medium may act in two ways. Firstly, by inhibiting thymidine phosphorylase (Budman & Pardee, 1967; Doskocil & Paces, 1968) and secondly, by allowing the uptake of thymine (derived from TdR) by providing a source of dRib-1-P. The first way seems to be the most important since deoxyadenosine prolongs the uptake of TdR in a pup$^-$ mutants; also the rate with which thymine appears in the medium is greatly retarded (Munch-Petersen, 1968b). A tpp$^-$ mutant is unable to degrade added TdR. Use of a dra$^-$ mutant for thymidine incorporation studies would result in thymine, derived from the thymidine, being incorpora-

ted, and not the thymidine itself. Caution should be used in the use of labelled TdR if a dra⁻ strain is used since growth inhibition may occur (see also Denhardt, 1969). However, the high cell concentrations usually used would tend to reduce growth inhibition and it may not be observed.

Wild-type strains of E.coli will not incorporate added thymine but will do so in the presence of deoxynucleosides. A thy⁻ mutant will take up added thymine without the addition of deoxynucleosides, but a high concentration (about 200 μM) is required. A drm⁻ or dra⁻ strain only requires about 20 μM thymine and would be the best choice of strain for studies using labelled thymine.

Finally, it may be noted that labelled TMP can be satisfactorily used as a thymine source in wild-type strains of E.coli (Breitman et al, 1967; Siminovitch & Graham, 1955): incorporation does not decrease due to catabolism, as with TdR. The work of Breitman was in fact inspired by a similar finding for dCMP (Lichtenstein et al, 1960). It was observed in this case that the pyrimidine nucleoside was incorporated intact into DNA, wihtout cleavage to give the free base. The phosphate moiety, however, was lost prior to penetration. The reason for the different behaviour of the nucleotide as opposed to the nucleoside is not known, but two hypotheses were advanced by Cohen's group. The first assumed that the dCMP was dephosphorylated to CdR which was then deaminated to UdR. It was then postulated that these reactions occur at the surface of the cell and that

deoxyuridine kinase is also surface localised whereas uridine phosphorylase was located inside the cell. Apart from the fact that the latter enzyme is known to be intracellularly localised (Heppel, 1967) no evidence is at present available on this hypothesis. However, in the case of TMP this hypothesis is made unlikely by the fact that thymidine phosphorylase is surface localised. The second hypothesis involved a phosphate exchange at the surface of the cell leading to the formation of a new nucleotide. This was considered less likely than the first hypothesis since most of the exogenous dCMP was directed to form thymidylate; this implied the reaction dCMP ——→ dUMP which is not known in E.coli. However, there is recently, evidence available which suggests that the majority of thymidylate in wild-type E.coli DNA is derived via cytidine compounds (Neuhard, 1968; Karlstrom & Larsson, 1967). The problem remains, however, why the cytosine and thymine residues of DNA are not at least equally derived from exogenous dCMP. Both hypotheses, therefore, seem equally unlikely at the present time.

(3) Thy⁻ Mutants and the Control of DNA Replication

The rate of DNA synthesis in E.coli is normally controlled by varying the frequency with which new rounds of replication are initiated (Maaløe & Kjeldgaard, 1966). The velocity with which a replication fork traverses the chromosome, at least for growth

rates between 22 and 70 minutes, is constant and is about 41 minutes, (see Helmstetter & Cooper, 1968; Helmstetter *et al*, 1968).

However, under certain conditions the time taken for a replicationfork to traverse the chromosome may become greater than 41 minutes. One of these conditions is when very low thymine concentrations are used in *E.coli* $15T^-$ (A. Zartisky, personal communication). Thus, at least in this strain of *E.coli*, the velocity with which a replication fork traverses the chromosome may be artificially limited by lowering the thymine concentration and hence limiting the size of the TTP pool.

OMISSION

References

ANDERSON, A., & R.A. COOPER (1969) The significance of ribokinase for ribose utilisation by Escherichia coli. Biochim. Biophys. Acta, 177, 163.

ARBER, W. (1968) Host-controlled restriction and modification of bacteriophage. 18th Symp. Soc. Gen. Microbiol., eds. L.V. Crawford and M.G.P. Stoker, p.295. Cambridge University Press.

EGGLESTON, L.V. & H.A. KREBS (1959) Permeability of Escherichia coli to ribose and ribose nucleotides. Biochem. J. 73, 264.

GARDNER, R., & A. KORNBERG (1967) Biochemical studies of bacterial sporulation and germination. V. Purine nucleoside phosphorylase of vegetative cells and spores of Bacillus cereus. J. Biol. Chem. 242, 2383.

GORINI, L. & J. BECKWITH (1966) Suppression. Ann. Rev. Microbiol., 20, 401.

JARETT, L. & R.W. HENDLER (1967) 2, 4-Dinitrophenol and azide as inhibitors of protein and ribonucleic acid synthesis in anaerobic yeast. Biochemistry, 6, 1693.

KALCKAR, H.M. (1947) Differential spectrophotometry of purine compounds by means of specific enzymes. I. Determination of hydroxypurine compounds. J.Biol. Chem. 167, 429.

LIN, E.C.C., J.P. KOCH, T.M. CHUSED, & S.E. JORGENSEN (1962) Utilization of L- -glycerophosphate by Escherichia coli without hydrolysis. Proc. Natl. Acad. Sci. 48, 2145

MALAMY, M.H. & B.L, HORECKER (1964) Rlease of alkaline phosphatase from cells of Escherichia coli upon lysozyme spheroplast formation. Biochemistry,3, 1889.

MOLLOY, A. & L.R. FINCH (1969) Uridine-5-monophosphate pyrophosphorylase activity from Escherichia coli. FEBS Letters 5, 211.

REMY, C.N. & S.H. LOVE (1968) Induction of adenosine deaminase in Escherichia coli. J.Bact., 96, 76.

ROSENBERG, H. & J.M. LA NAUZE (1968) The isilation of a mutant of Bacillus cereus deficient in phosphate uptake. Biochim. Biophys. Acta 156, 381.

YARMOLINSKY, M.B., H. WIESMEYER, H.M. KALCHAR & E. JORDAN (1959) Hereditary defects in galactose metabolism in Escherichia coli mutants. II. Galactose-induced sensitivity. Proc. Natl. Acad. Sci., 45, 1786

REFERENCES

ADELBERG, E.A., M. MANDEL & G.C.C. CHEN (1965) Optimal conditions for mutagenesis by N-methyl-N'-nitrosoguanidine in Escherichia coli K12. Biochem.Biophys. Res. Commun., 18, 788.

AHMAD, S.I., P.T. BARTH & R.H. PRITCHARD (1968) Properties of a mutant of Escherichia coli lacking purine nucleoside phosphorylase. Biochim. Biophys. Acta, 161, 581.

AHMAD, S.I. & R.H. PRITCHARD (1969) A map of four genes specifying enzymes involved in catabolism of nucleosides and deoxynucleosides in Escherichia coli. Molec. Gen. Genetics, 104, 351.

ALBAUM, H.G. & W.W. UMBREIT (1947) Differentiation between ribose-3-phosphate and ribose-5-phosphate by means of the orcinol-pentose reaction. J.Biol.Chem., 167, 369.

ALIKHANIAN, S.E., T.S. ILJINA, E.S. KALIAEVA, S.V. KAMENEVA, & U.U. SUKHODOLEC (1966) A genetical study of thymineless mutants of E.coli K12. Genet. Res., 8, 83.

ANDERSON, H. (1946) Growth requirements of virus resistant mutants of a bacterial strain. Proc. Natl. Acad. Sci., 32, 120.

BARKULIS, I.L. (1949) Growth inhibition of Eberthalla typhosa by certain carbohydrates and its release by mutation. J. Bact., 58, 103.

BARNER, H.D. & COHEN, S.S. (1959) Virus induced acquisition of metabolic function. IV. Thymidylate synthetase in thymine-requiring E.coli infected by T2 and T5 bacteriophages. J.Biol. Chem., 234, 2987.

BARTH, P.T. (1968) Genetic and biochemical studies on the control of DNA replication in bacteria. Ph.D. Thesis (Leicester).

BARTH, P.T., I.R. BEACHAM, S.I. AHMAD & R.H. PRITCHARD (1968) The inducer of the deoxynucleoside phosphorylases and deoxyribo-aldolase in Escherichia coli. Biochim. Biophys. Acta, 161, 554.

BEACHAM, I.R., A. EISENSTARK, P.T. BARTH & R.H. PRITCHARD (1968a) Deoxynucleoside-sensitive mutants of Salmonella typhimurium. Molec. Gen. Genetics, 102, 112.

BEACHAM, I.R., P.T. BARTH & R.H. PRITCHARD (1968b). Constitutivity of thymidine phosphorylase in deoxyriboaldolase negative strains: dependence on thymine requirement and concentration. Biochim. Biophys. Acta, 166, 589.

BERTANI, L.E., A. HÄGGMARK & P. REICHARD (1963) Enzymatic synthesis of deoxyribonucleotides. II. Formation and interconversion of deoxyuridine phosphates. J.Biol. Chem., 238, 3407.

BISWAS, C., J. HARDY & W.S. BECK (1965) Release of repressor control of ribonucleotide reductase by thymine starvation. J. Biol. Chem. 240, 3631.

BOCK, A. & F.C. NEIDHARDT (1966a) Isolation of a mutant of Escherichia coli with a temperature-sensitive fructose-1,6-diphosphate aldolase activity. J.Bact., 92, 464.

BOCK, A. & F.C. NEIDHARDT (1966b) Properties of a mutant of Escherichia coli with a temperature-sensitive fructose-1,6-diphosphate aldolase. J.Bact., 92, 470.

BOLTON, E.T. & A.M. REYNARD (1954) Utilisation of purine and pyrimidine compounds in nucleic acid synthesis by Escherichia coli. Biochim. Biophys. Acta, 13, 381.

BOYCE, R.P. & R.B. SETLOW (1962) A simple method of increasing the incorporation of thymidine into the deoxyribonucleic acid of Escherichia coli. Biochim. Biophys. Acta, 61, 688

BREITMAN, T.R. & R.M. BRADFORD (1964) The induction of thymidine phosphorylase and excretion of deoxyribose during thymine starvation. Biochem. Biophys. Res. Commun., 17, 786.

BREITMAN, T.R. & R.M. BRADFORD (1967a) The absence of deoxyribo-aldolase activity in a thymineless mutant of Escherichia coli strain 15: A possible explanation for the low thymine requirement of some bacterial strains. Biochim. Biophys. Acta, 138, 217.

BREITMAN, T.R. & R.M. BRADFORD (1967b) Metabolism of thymineless mutants of Escherichia coli. I. Absence of thymidylate synthetase activity and growth characteristics of two sequential thymineless mutants. J.Bact., 93, 845.

BREITMAN, T.R., R.M. BRADFORD & W. DILWORTH CANNON Jr. (1967) Use of exogenous deoxythymidylic acid to label the deoxyribonucleic acid of growing wild-type Escherichia coli. J.Bact., 93, 1471.

BREITMAN, T.R. & R.M. BRADFORD (1968) Inability of low thymine requiring mutants of Escherichia coli lacking phosphodeoxyribomutase to be induced for deoxythymidine phosphorylase and deoxyriboaldolase. J.Bact., 95, 2434.

BROCKMAN, R.W. & L.A. HEPPEL (1968) On the localisation of alkaline phosphatase and cyclic phosphodiesterase in Escherichia coli. Biochemistry, 7, 2554.

BROWN, N.C. & P. REICHARD (1969a) Ribonucleoside diphosphate reductase. Formation of active and inactive complexes of proteins B1 and B2. J.Mol.Biol., 46, 25.

BROWN, N.C. & P. REICHARD (1969b) Role of effector binding in allosteric control of ribonucleoside diphosphate reductase. J.Mol. Biol, 46, 39.

BUDMAN, D.R. & A.B. PARDEE (1967) Thymidine and thymine incorporation into deoxyribonucleic acid: Inhibition and repression by uridine of thymidine phosphorylase of Escherichia coli J.Bact., 94, 1546.

BURTON, K. (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of desoxyribonucleic acid. Biochem. J., 62, 315.

COHEN, S.S. & H.D. BARNER (1957) The conversion of 5-methyldeoxycytidine to thymidine *in vitro* and *in vivo*. J.Biol.Chem., 226, 631.

COOPER, R.A., S. PERRY & T.R. BREITMAN (1966) Pyrimidine metabolism in human leucocytes. I. Contribution of exogenous thymidine to DNA-thymine and its effect on thymine nucleotide synthesis in leukemic leukocytes. Cancer Res., 26, 2267.

CORDONNIER, C. & BERNARDI, G. (1965) Localisation of *E.coli* endonuclease I. Biochem. Biophys. Res. Commun., 20, 555.

COZZARELLI, N.R., J.P. KOCH & E.C.C. LIN (1965) Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J.Bact., 90,1325.

CRAWFORD, L.V. (1958) Thymine metabolism in strains of *Escherichia coli*. Biochim. Biophys. Acta, 30, 528.

CRAWFORD, I., A. KORNBERG & E.S. SIMMS (1957) Conversion of uracil and orotate to uridine 5'-phosphate by enzymes in Lactobacilli. J. Biol. Chem., 226, 1093.

DALE, B. & G.R. GREENBERG (1967) Genetic mapping of a mutation in *Escherichia coli* showing reduced activity of thymidine phosphorylase. J.Bact., 94, 778.

DAMADIAN, R. (1967) Abnormal phosphorous metabolism in a potassium transport mutant of *Escherichia coli*. Biochim. Biophys. Acta, 135, 378.

DENHARDT, D.T. (1969) Formation of ribosyl thymine in Escherichia coli. Studies on pulse labelling with thymine and thymidine. J.Biol.Chem., 244, 2710.

DILWORTH CANNON Jr. & T.R. BRETIMAN (1967) Control of deoxynucleotide biosynthesis in Escherichia coli. I. Decrease of pyrimidine deoxynucleotide biosynthesis in vivo in the presence of deoxythymidylate. Biochemistry, 6, 810.

DILWORTH CANNON Jr. & T.R. BREITMAN (1968) Control of deoxynucleotide biosynthesis in Escherichia coli. II. Effect of deoxythymidylate on the biosynthesis of both deoxynucleotides and ribonucleotide reductase. Arch. Biochem. Biophys. 127, 534.

DIXON, G.H. & H.L. KORNBERG (1959) Assay methods for key enzymes of the glyoxylate cycle. Biochem. J., 72, 3P.

DONE, J., C.D. SHOREY, J.P. LOKE & J.K. POLLAK (1965) The cytochemical localisation of alkaline phosphatase in Escherichia coli at the electron microscope level. Biochem. J., 96, 27C.

DOSKOCIL, J. & V. PACES (1968) The effect of nucleosides on the phosphorolysis of thymidine by normal and phage-infected cells of E.coli. Biochem. Biophys. Res. Commun., 30, 153.

DVORAK, H.F., R.W. BROCKMAN & L.A. HEPPEL (1967) Purification and properties of two acid phosphatase fractions isolated from osmotic shock fluid of Escherichia coli. Biochemistry, 6, 1743.

ENGLESBERG, E., R.L. ANDERSON, R. WEISBERG, N. LEE, P. HOFFEE, A. HUTTENHAUER & H. BOYER (1962) L-arabinose-sensitive L-ribulose

5-phosphate 4-epimerase-deficient mutants of Escherichia coli. J.Bact., 84, 137.

ENGLESBERG, E. & L.S. BARON (1959) Mutation of L-rhamnulose resistance and transduction to L-rhamnose utilisation in Salmonella typhosa. J.Bact., 78, 675.

FANGMAN, W.L. & A. NOVICK (1966) Mutant bacteria showing efficient utilisation of thymidine. J.Bact., 91, 2390.

FANGMAN, W.L. (1969) Specificity and efficiency of thymidine incorporation in Escherichia coli lacking thymidine phosphorylase. J.Bact., 99, 681.

FLAKS, J.G. (1963) 5-phosphoribose pyrophosphokinase. Meth. Enzymol. 6, 158.

FORSTER, E. & A.W. HOLLDORF (1965) Abstracts 2nd FEBS Meeting Vienna) p.146.

FRAENKEL,D.G. (1968) The accumulation of glucose-6-phosphate from glucose and its effect in an E.coli mutant lacking phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. J.Biol. Chem., 243, 6451.

FRIEDKIN, M. & H.M. KALCKAR (1950) Deoxyribose-1-phosphate. I. The phosphorolysis and resynthesis of purine deoxyribonucleoside. J.Biol. Chem., 184, 437.

FRIEDKIN, M. & H. KALCKAR (1961) Nucleoside phosphorylases. In The Enzymes, ed. Boyer, Hardy, Myrbäck. Vol.5.

FRIEDMAN, S.B. & P. MARGOLIN (1968) Evidence for an altered operator

specificity: catabolite repression control of the leucine operon in Salmonella typhimurium. J.Bact., 95, 2263.

GAREN, A. & C. LEVINTHAL (1960) A fine structure genetic and chemical study of the enzyme alkaline phosphatase of E.coli. 1. Purification and characterisation of alkaline phosphatase. Biochim. Biophys. Acta, 38, 470.

GILLESPIE, D., M. DEMEREC & H. ITIKAWA (1968) Appearance of double mutants in aged cultures of Salmonella typhimurium cysteine requiring strains. Genetics, 59, 433.

GLASER, L., A. MELO & R. PAUL (1967) Uridine diphosphate sugar hydrolase. Purification of enzyme and protein inhibitor. J.Biol. Chem., 242, 1944.

GLOVER, S.W. & J. SCHELL (1966) On the localisation of a factor responsible for host controlled restriction in E.coli K (P1) Genet. Res. (Camb) 7, 277.

GOSS, W.A., W.H. DEITZ & T.M. COOKE (1964) Mechanism of action of nalidixic acid on E.coli. J.Bact., 88, 1112.

HARRISON, A.P. (1965) Thymine incorporation and metabolism by various classes of thymineless bacteria. J.Gen.Microbiol., 41, 321.

HELMSTETTER, C.E. & S. COOPER (1968) DNA synthesis during the division cycle of rapidly growing Escherichia coli B/r. J.Mol.Biol., 31, 507.

HELMSTETTER, C., S. COOPER, O. PIERUCCI & E.REVELAS (1968) On the bacterial life sequence. Cold Spring Harbor Symp. Quant. Biol., 33, 809.

HEPPEL, L.A. (1967) Selective release of enzymes from bacteria. Science, 156, 1451.

HEREDIA, C.F. & A. SOLS (1964) Metabolic studies with 2-deoxyhexoses II. Resistance to 2-deoxyglucose in a yeast mutant. Biochim. Biophys. Acta, 86, 224.

HIRAGA, S., K. IGARASHI, & T. YURA (1967) A deoxythymidine kinase deficient mutant of Escherichia coli. I. Isolation and some properties. Biochim. Biophys. Acta, 145, 41.

HOFFEE, P.A. (& B.C. ROBERTSON) (1969a) 2-Deoxyribose gene-enzyme complex in Salmonella typhimurium. I. Isolation and enzymatic characterisation of 2-deoxyribose-negative mutants. J. Bact., 95, 449.

HOFFEE, P.A. (1968b) 2-Deoxyribose-5-phosphate aldolase of Salmonella typhimurium: Purification and properties. Arch. Biochem. Biophys. 126, 795.

HOFFEE, P.A. & B.C. ROBERTSON (1969) 2-Deoxyribose gene-enzyme complex in Salmonella typhimurium. Regulation of phosphodeoxyribomutase. J.Bact, 97, 1386.

HOFFMANN, C.E. & J.O. LAMPEN (1952) Products of deoxyribose degradation by Escherichia coli. J. Biol. Chem., 198, 885.

HOTCHKISS, R.D. (1948) The quantitative separation of purines, pyrimidines and nucleosides by paper chromatography. J.Biol. Chem., 175, 315.

IMADA, A. & S. IGARASI (1967) Ribosyl and deoxyribosyl transfer by bacterial enzyme systems. J. Bact., 94, 1551.

IPATA, P.L. (1968) Sheep brain 5'-nucleotidase. Some enzymatic properties and allosteric inhibition of nucleoside triphosphates. Biochemistry, 7, 507.

JAYARAMAN, K., B. MÜLLER-HILL & H.V. RICHENBERG (1966) Inhibition of the synthesis of β-galactosidase in Escherichia coli by 2-nitrophenyl-β-D-fucoside. J.Mol.Biol., 18, 339.

KARLSTRÖM, O. & A. LARSSON (1967) Significance of ribonucleotide reduction in the biosynthesis of deoxyribonucleotides in Escherichia coli. Europ. J. Biochem., 3, 164.

KARLSTRÖM, O. (1968) Mutants of Escherichia coli defective in ribonucleoside and deoxyribonucleoside catabolism. J. Bact., 95, 1069.

KAMMEN, H.O. (1967a) Enzymes of deoxyribose metabolism in E.coli. Fed. Proc., 26, 809.

KAMMEN, H.O. (1967b) Thymine metabolism in Escherichia coli. I. Factors involved in utilisation of exogenous thymine. Biochim. Biophys. Acta, 134, 301.

KATZ, J. & R. ROGNSTAD (1967) The labelling of pentose phosphate from glucose-C^{14} and estimation of the rates of transaldolase, transketolase, the contributions of pentose cycle and ribose phosphate synthesis. Biochemistry, 6, 2227.

KJELDGAARD, N.O. (1961) The kinetics of ribonucleic acid and protein formation in Salmonella typhimurium during the transition between different states of balanced growth. Biochim. Biophys. Acta, 49, 64.

KLENOW, H. (1959) On the effect of some adenine derivatives on the incorporation in vitro of isotopically labelled compounds into the nucleic acids of Ehrlich ascites tumor cells. Biochim. Biophys. Acta, 35, 412.

KLENOW, H. (1962) Further studies on the effect of deoxyadenosine on the accumulation of deoxyadenosine triphosphate and inhibition of deoxyribonucleic acid synthesis in Ehrlich ascites tumor cells in vitro. Biochim. Biophys. Acta, 61, 885.

KOCH, A.L. & W.A. LAMONT (1956) The metabolism of methyl purines by Escherichia coli II. Enzymatic studies. J.Biol.Chem., 219, 189.

KORNBERG, A., I. LIEBERMAN & E.S. SIMMS (1955) Enzymatic synthesis and properties of 5-phosphoribosylpyrophosphate. J.Biol.Chem., 215, 389.

KORNBERG, A. (1962) On the metabolic significance of phosphorolytic and pyrophosphorolytic reactions. Horizons in Biochemistry (Ed. M. Kasha & B. Pullman; Academic Press, New York), p.251.

KORNBERG, H.L. (1967) The regulation of anaplerotic enzymes in E.coli. Bull. Soc. Chim. Biol., 49, 1479.

KOVAC, L. & S. KUZELA (1966) Effect of uncoupling agents and azide on the synthesis of β-galactosidase in aerobically grown Escherichia coli. Biochim. Biophys. Acta, 127, 355.

KRAKOFF, I.H., N.C. BROWN & P. REICHARD (1968) Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Canc. Res., 28, 1559.

KRENITSKY, T.A., M. BARCLAY & J.A. JACQUEZ (1964) Specificity of mouse uridine phosphorylase. J.Biol.Chem., 239, 805.

KURAHASHI, K. & A.J. WAHBA (1958) Interference with growth of certain Escherichia coli mutants by galactose. Biochim. Biophys. Acta, 30, 298.

LAMPEN, J.O. (1952) Enzymatic studies on the metabolism of nucleosides and their components in microorganisms, in Phosphorous Metabolism, Vol. II, p.363, ed. W.D. McElroy & H.B. Glass. (Johns Hopkins Press, Baltimore)

LARSSON, A. & P. REICHARD (1967) Enzymatic reduction of ribonucleotides, in Progress in Nucleic Acid Research, Vol. 7, p.203, ed. J.W.Davidson & W.H. Cohen. (Academic Press, New York).

LEHMAN, I.R., M.J. BESSMAN, E.S. SIMMS & A. KORNBERG (1964) Enzymatic synthesis of deoxyribonucleic acid. I. Preparation of substrates and partial purification of an enzyme from Escherichia coli. J.Biol. Chem., 239, 269.

LICHTENSTEIN, J., H.D. BARNER & S.S. COHEN (1960) The metabolism of exogenously supplied nucleotides by Escherichia coli. J.Biol. Chem., 235, 457.

LIEVE, L. (1968) Studies on the permeability change produced in coliform bacteria by ethylene-diaminetetraacetate. J.Biol. Chem., 243, 2373.

LOMAX, M.S. & G.R. GREENBERG (1968) Characteristics of the deo operon: Role in thymine utilisation and sensitivity to deoxyribonucleosides. J.Bact., 96, 501.

LONG, C.W. & A.B. PARDEE (1967) Cytidine triphosphate synthetase of Escherichia coli B. I. Purification and properties. J.Biol. Chem., 242, 4715.

LOWRY, O.H., N.J. ROSEBROUGH, A.L. FARR & R.J. RANDALL (1951) Protein measurement with the Folin phenol reagent. J.Biol. Chem., 193, 265.

MAALØE,O. & N.O. KJELDGAARD (1966) Control of Macromolecular Synthesis. (W.A. Benjamin Inc.)

MAGASANIK, B. (1961) Catabolite repression. Cold Spring Harbor Symp. Quant. Biol., 26, 249.

MAGASANIK, B. (1962) Biosynthesis of purine and pyrimidine nucleotides, in The Bacteria, vol III, p.295, ed. Gunsalus & Stanier. (Academic Press, New York).

MANDELSTAM, J. (1961) Induction and repression of β-galactosidase in non-growing Escherichia coli. Biochem. J. 79, 489.

MANGIAROTTI, G., D. APIRION & D. SCHLESSINGER (1966) Selection of sucrose-dependent Escherichia coli to obtain envolope mutants and fragile cultures. Science, 153, 892.

MANSON, L.A., & J.O. LAMPEN (1951) The metabolism of hypoxanthine deoxyriboside in animal tissues. J.Biol.Chem., 191, 95.

MANTSAVINOS, R. & S. ZAMENHOF (1961) Pathways for the biosynthesis of thymidylic acid in bacterial mutants. J.Biol. Chem., 236, 876.

MARUYAMA, H. & D. MIZUNO (1965) The participation of ribonuclease in the degradation of Escherichia coli ribosomal ribonucleic acid as revealed by oligonucleotide accumulation in the phosphorus-deficient stage. Biochim. Biophys. Acta, 108, 593.

McFALL, E. (1964) Pleiotropic mutations in the D-serine deaminase system of E.coli. J.Mol.Biol., 9, 754.

McFALL, E. (1967) Dominance studies with stable merodiploids in the D-serine deaminase system of Escherichia coli K-12. J. Bact., 94, 1982.

MELO, A. & L. GLASER (1966) Nucleotide diphosphate hexose pyrophosphatases.. Biochem. Biophys. Res. Commun., 22, 524.

MINEEVA, L.A. & T.S. IL'INA (1969) Thymidylate biosynthesis in thymine dependent mutants of Escherichia coli K-12. Biokhimiya, 34, 17 (English translation).

MITCHELL, P. (1961) Approaches to the analysis of specific membrane transport, in Biological Structure and Function, Vol. II, p.581, ed. T.W.Goodwin & O. Lindberg. (Academic Press, New York).

MOAT, A.G. & FRIEDMAN, H. (1960) The biosynthesis and interconversion of purines and their derivatives. Bacteriol. Rev., 24, 309.

MOSES, V. & M.D. YUDKIN (1968) Catabolite repression in Escherichia coli. A study of two hypotheses. Biochem. J., 110, 135.

MUNCH-PETERSEN, A. (1960) Formation in vitro of deoxyadenosine triphosphate from deoxyadenosine in Ehrlich ascites cells. Biochem. Biophys. Res. Commun., 3, 392.

MUNCH-PETERSEN, A. (1967) Thymidine breakdown and thymine uptake in different mutants of Escherichia coli. Biochim. Biophys. Acta, 142, 228.

MUNCH-PETERSEN, A. (1968a) Thymineless mutants of Escherichia coli with deficiencies in deoxyribomutase and deoxyriboaldolase. Biochim. Biophys. Acta, 161, 279.

MUNCH-PETERSEN, A. (1968b) On the catabolism of deoxyribonucleosides in cells and cell extracts of Escherichia coli. Europ. J. Biochem., 6, 432.

MORRIS, N.R. & G.A. FISCHER (1960) Studies concerning inhibition of the synthesis of deoxycytidine by phosphorylated derivatives of thymidine. Biochim. Biophys. Acta, 42, 183.

NELSON, D.J. & C.E. CARTER (1969) Purification and characterisation of thymidine 5'-monophosphate kinase from Escherichia coli B. J.Biol.Chem., 244, 5254.

NEU, H.C. (1967a) The 5'-nucleotidase of Escherichia coli. I. Purification and properties. J.Biol.Chem., 242, 3896.

NEU, H.C. (1967b) The 5'-nucleotidase of Escherichia coli. II. Surface localisation and purification of the Escherichia coli 5'-nucleotidase inhibitor. J. Biol.Chem., 242, 3905.

NEU, H.C. (1968) The 5'-nucleotidases and cyclic phosphodiesterases (3'-nucleotidases) of the Enterobacteriaceae. J.Bact., 95,1732.

NEU, H.C. & L.A. HEPPEL (1965) The release of enzymes from Escherichia coli by osmotic shock and during the formation of spheroplasts. J. Biol.Chem., 240, 3685.

NEUHARD, J. & A. MUNCH-PETERSEN (1966) Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of Escherichia coli, during thymine starvation. II. Changes in the amounts of deoxycytidine triphosphate and deoxyadenosine triphosphate in Escherichia coli 15 $T^{-}A^{-}U^{-}$. Biochim. Biophys. Acta, 114, 61.

NEUHARD, J. (1966) Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of Escherichia coli during thymine starvation. III. On the regulation of the deoxyadenosine triphosphate and deoxycytidine triphosphate pools of Escherichia coli. Biochim. Biophys. Acta, 129, 104.

NEUHARD, J. (1967) Studies on the acid-soluble nucleotide pool in Escherichia coli IV. Effects of hydroxyurea. Biochim. Biophys. Acta, 145, 1.

NEUHARD, J. & J. INGRAHAM (1968) Mutants of Salmonella typhimurium requiring cytidine for growth. J.Bact., 95, 2431.

NEUHARD, J. (1968) Pyrimidine nucleotide metabolism and pathways of thymidine triphosphate biosynthesis in Salmonella typhimurium J.Bact., 96, 1519.

NISONSON, I., M. TANNENBAUM & H.C. NEU (1969) Surface localisation of Escherichia coli 5'-nucleotidase by electron microscopy. J.Bact., 100, 1083.

NOSSAL, N.G. & L.A. HEPPEL (1966) The release of enzymes by osmotic shock from Escherichia coli in exponential phase. J. Biol. Chem., 241, 3055.

OKADA, T., K. YANAGISAWA & F.J. RYAN (1961) A method for securing thymineless mutants from strains of E.coli. Z. Vererbungsl. 92, 403.

OKADA, T. (1966) Mutational site of the gene controlling quantitative thymine requirement in Escherichia coli K12. Genetics, 54, 1329.

OKAZAKI, R. & A. KORNBERG (1964) Deoxythymidine kinase of Escherichia coli. I. Purification and some properties of the enzyme. J.Biol. Chem., 239, 269.

OVERATH, P. (1968) Control of basal level activity of β-galactosidase in Escherichia coli. Molec. Gen. Genetics, 101, 155.

PAEGE, L.M. & F. SCHLENK (1952) Bacterial uracil riboside phosphorylase. Arch. Biochem. Biophys. 40, 42.

PARDEE, A.B., F. JACOB & J. MONOD (1959) The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by E.coli. J.Mol.Biol., 1, 165.

PETERSEN, R.H., J. BONIFACE & A.L. KOCH (1967) Energy requirements, interactions and distinctions in the mechanisms for transport of various nucleosides in Escherichia coli. Biochim. Biophys. Acta, 135, 771.

PONTIS, H., G. DEGERSTEDT & P. REICHARD (1961) Uridine and deoxyuridine phosphorylases from Ehrlich ascites tumor. Biochim. Biophys. Acta, 51, 138.

RACHMELER, M., J. GERHART & J. ROSNER (1961) Limited thymidine uptake in Escherichia coli due to an inducible thymidine phosphorylase. Biochim. Biophys. Acta, 49, 222.

RACKER, E. (1952) Enzymatic synthesis and breakdown of deoxyribose phosphate. J.Biol.Chem., 196, 347.

RAZZELL, W.E. & P. CASSHYAP (1964) Substrate specificity and induction of thymidine phosphorylase in Escherichia coli. J.Biol. Chem., 239, 1789.

RAZZELL, W.E. & H.G. KHORANA (1958) Purification and properties of a pyrimidine deoxyriboside phosphorylase from Escherichia coli. Biochim. Biophys. Acta, 28, 562.

REICHARD, P. (1967) The Biosynthesis of Deoxyribose. John Wiley & Sons Inc. New York.

ROBERTS, R.B., P.H. ABELSON, D.B. COWIE, E.T. BOLTON, R.J. BRITTEN (1963) Studies of biosynthesis in E.coli. Carnegie Institute of Washington.

ROEPKE, R.R.,& P.E. MERCER (1947) Lethal and sublethal effects of X-rays on Escherichia coli as related to the yield of biochemical mutants. J.Bact., 54, 731.

ROEPKE, R.R. (1967) Relation between different thymineless mutants derived from E.coli. J.Bact., 93, 1188.

ROODYN, D.B. & H.G. MANDEL (1960) A simple membrane fractionation method for determining the distribution of radioactivity in chemical fractions of Bacillus cereus. Biochim. Biophys. Acta, 41, 80.

ROSENKRANTZ, H.S., A.J. GARRO, J.A. LEVY & H.S. CARR (1966) Studies with hydroxyurea. I. The reversible inhibition of bacterial DNA synthesis and the effect of hydroxyurea on the bacteriocidal action of streptomycin. Biochim. Biophys. Acta, 114, 501.

SABLE, H.Z. (1966) Biosynthesis of ribose and deoxyribose. Adv. Enzymol., 28, 391.

SCHLESINGER, M.J. (1968) Secretion of alkaline phosphatase subunits by spheroplasts of Escherichia coli. J.Bact., 96, 727.

SCHLESINGER, M.J. & R. OLSEN (1968) Expression and localisation of Escherichia coli alkaline phosphatase synthesised in Salmonella typhimurium cytoplasm. J.Bact., 96, 1601.

SCHELSINGER, S., P. SCOTTO & B. MAGASANIK (1965) Exogenous and endogenous induction of the histidine-degrading enzymes in Aerobacter aerogenes. J.Biol.Chem., 240, 4331.

SIMONI, R.D., M. LEVINTHAL, F.D. KUNDIG, W. KUNDIG, B. ANDERSON, P.E. HARTMAN & S. ROSEMAN (1967) Genetic evidence for the role of a bacterial phosphotransferase system in sugar transport. Proc. Natl. Acad. Sic., 58, 1963.

SIMINOVITCH, L. & A.F. GRAHAM (1955) Synthesis of nucleic acids in Escherichia coli. Canad. J. Microbiol., 1, 721.

SLATER, E.C. (1953) Mechanism of phosphorylation in the respiratory chain. Nature, 172, 975.

SMITH, B.J. (1964) Ph.D. Thesis,University of Oxford.

SMITH, D.H. & B.D. DAVIS (1967) Mode of action of novobiocin in Escherichia coli. J.Bact., 93, 71.

SMITH, M. & H.G. KHORANA (1963) Preparation of nucleotides and derivatives. Meth. Enzymol. Vol. III, p.659.

SPICER, S.S., B.K. WETZEL & L.A. HEPPEL (1966) Fine structural cytochemistry of several phosphatases in E.coli. Fed.Proc. 25,539.

STACEY, K.A. & E. SIMPSON (1965) Improved method for the isolation of thymine-requiring mutants of Escherichia coli. J.Bact., 90, 554.

SWENSON, P.A. & R.B. SETLOW (1966) Effects of ultraviolet radiation on macromolecular synthesis in E.coli. J.Mol.Biol., 15, 201.

TAYLOR, A.L. & C.D. TROTTER (1967) Revised linkage map of Escherichia coli. Bacteriol. Rev., 31, 332.

TORRIANI, A. (1960) Influence of inorganic phosphate in the formation of phosphatases by Escherichia coli. Biochim. Biophys. Acta, 38, 460.

WANG, R.J. & M.L. MORSE (1968) Carbohydrate accumulation and metabolism in Escherichia coli. 1. Description of pleiotropic mutants. J.Mol.Biol., 32, 59.

WANG, C.H., I. STERN, C.M. GILMOUR, S. KLUNGSAYR, D.J. REED, J.J. BIALY, B.E. CHRISTENSEN, & U.H. CHELDELIN (1958) Comparative study of glucose catabolism by the radio-respirometric method. J. Bact., 76, 207.

WARD, J.B. & L. GLASER (1968) An E.coli mutant with cryptic UDP-sugar hydrolase and altered metabolite regulation. Biochem. Biophys. Res. Commun., 31, 671.

WARD, J.B. & L. GLASER (1969) Turnover of UDP-sugars in E.coli mutants with altered UDP-sugar hydrolase. Arch. Biochem. Biophys. 134, 612.

WU, H.C.P. & H.M. KALCKAR (1966) Endogenous induction of the galactose operon in Escherichia coli K12. Proc. Natl. Acad. Sci., 55, 622.

YUAN, R. & M. MESELSON (1968) DNA restriction enzyme from E.coli. Nature, 217, 1110.

ZIMMERMAN, M. (1964) Deoxyribosyl transfer. II. Nucleoside: pyrimidine deoxyribosyltransferase activity of three partially purified thymidine phosphorylases. J.Biol. Chem., 239, 2622.

ZWAIG, N. & E.C.C. LIN (1966) Feedback inhibition of glycerol kinase, a catabolic enzyme in Escherichia coli. Science 153, 755.

Acknowledgements

I would like to express my deep gratitude to Professor R.H. Pritchard for his expert guidance and teaching during this work.

I would also like to thank many other members (too numerous to mention) of both the Genetics and Biochemistry Departments for their comments and help at various times.

My thanks are also due to Margaret Peake for typing this thesis and to my wife, Kathryn, who drew most of the figures.

PUBLICATIONS

Barth, P.T., I. Beacham, S.I. Ahmad & R.H. Pritchard (1968) Properties of bacterial mutants defective in the catabolism of deoxynucleosides. Biochem. J. 106, 36P.

Barth, P.T., I.R. Beacham, S.I. Ahmad & R.H. Pritchard (1968) The inducer of the deoxynucleoside phosphorylases and deoxyriboaldolase in Escherichia coli. Biochim. Biophys. Acta, 161, 554.

Beacham, I.R., A. Eisenstark, P.T. Barth & R.H. Pritchard (1968) Deoxynucleoside-sensitive mutants of Salmonella typhimurium. Molec. Gen. Genetics, 102, 112.

Beacham, I.R., P.T. Barth & R.H. Pritchard (1968) Constitutivity of thymidine phosphorylase in deoxyriboaldolase negative strains: dependence on thymine requirement and concentration. Biochim. Biophys. Acta, 166, 589.

Beacham, I.R. (1969) A new assay for phosphodeoxyribomutase: surface localisation of the enzyme. Biochim. Biophys. Acta, 191, 158.