STUDIES ON THE METABOLIC CHANGES ACCOMPANYING BACTERIOPHAGE INFECTION

A thesis submitted for the
Degree of Doctor of Philosophy
at the University of Oxford

by

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ABSTRACT

Introduction

The infection of bacteria by viruses provides a simple system with which to study the basic processes of viral infection. Until about 1960 detailed bacteriophage research was restricted almost entirely to bacteriophages of Escherichia coli. Since 1960 a number of bacteriophages of Bacillus subtilis have been investigated. B. subtilis was chosen as a suitable host organism because it will grow in a simple chemically-defined medium, because auxotrophic mutants may be isolated, and because such mutants may be genetically mapped by transformation.

A group of virulent B. subtilis phages which are of particular interest contain hydroxymethyluracil in place of thymine in their DNA. They are comparable with the extensively-investigated T-even coliphages which contain hydroxymethylcytosine in place of cytosine in their DNA. The presence of the unusual base provides a means of identifying phage DNA in the infected cell, and of studying the phage-induced alterations of metabolism necessary for the biosynthesis of the unusual nucleotide. The virulent phages of B. subtilis have therefore been further investigated.

By analogy with the T-even coliphages the *B. subtilis* phages were expected to induce two special classes of enzymes after infection. These are (i) enzymes involved in the biosynthesis of hydroxymethyluracil, and (ii) enzymes which prevent the accumulation of thymidine triphosphate (dTTP) and its subsequent incorporation into phage DNA. The biosynthesis of hydroxymethyluracil is satisfactorily accounted for by the presence, in phage-infected cells, of an enzyme which hydroxymethylates deoxyuridylic acid (dUMP). A second phage-induced enzyme which deaminates deoxycytidylic acid may augment the normal intracellular supply of dUMP and thus control the relative levels of phosphorylated derivatives of deoxycytidine and hydroxymethyldeoxyuridine.

There is less general agreement about the phage-induced control of thymine biosynthesis. Different *B. subtilis* phages have been found to induce (i) an enzyme which hydrolyses dTTP to thymidylic acid (dTMP); (ii) an enzyme which hydrolysis dTMP to thymidine; and (iii) a rapid disappearance of the enzyme (thymidylate synthetase), which catalyses a methylation of dUMP to form dTMP. One of the aims of the present investigation was to determine whether one phage induced more than one of the possible mechanisms for preventing the incorporation of thymine into phage DNA.

The second object of the work was to investigate the phage Øe-induced inactivation of dTMP synthetase. The rapidity of the inhibition and the circumstances of its occurrence suggested the operation of an unusual form of control of enzyme activity. The control of metabolism by agents which inhibit existing enzymes is well-known in the case of feed-back inhibition, where a small molecular weight substance is implicated. The possibility that enzymes may be inactivated by alternative means such as protein inhibitors is a neglected aspect of metabolic control. A few examples of enzymes disappearing rapidly as a result of phage infection or as a result of the repression of enzyme synthesis are known and in some cases a protein inhibitor is implicated. The phage-induced inactivation of dTMP synthetase has also been shown to be caused by a protein inhibitor. The properties of the inhibitor and the mechanism of its interaction with dTMP synthetase.

The inhibition of dTMP synthetase was detectable 5 min after the addition of phage Øe and less than 10% of the original activity remained at 12 min after infection. If protein synthesis was inhibited no loss of dTMP synthetase occurred. An inhibitor of dTMP synthetase was detected in extracts from phage-infected cells. It had the properties of

a protein. It was heat-labile, non-dialysable, excluded from Sephadex G25, precipitated by ammonium sulphate and destroyed by digestion with trypsin. The amount of inhibitor present in extracts from phage-infected cells increased for at least the first 25 min of phage infection. It therefore behaved as if it were a phage-induced protein.

In order to study the mechanism of the inhibition process, dTMP synthetase was purified 30 fold from uninfected B. subtilis using ammonium sulphate fractionation, DEAE-cellulose chromatography and gel filtration. The inhibitor was purified 10-20 fold from B. subtilis cells which were harvested 25 min after infection. The purification of the inhibitor involved ammonium sulphate fractionation and DEAE-cellulose chromatography, and was complicated by the instability of the inhibitor.

The interaction of dTMP synthetase and the inhibitor was studied most thoroughly using their respective ammonium sulphate fractions. However preliminary results with the more highly purified samples of both proteins tended to confirm the results obtained with the less pure system. In order to detect and assay the inhibitor it was necessary to preincubate it with a sample of dTMP synthetase at 37^{o} for a short period of time (10 min usually) before assaying the

mixture for residual dTMP synthetase activity. The interaction between the two proteins was not instantaneous. Thymidylate synthetase activity continued to decrease for at least 30 min after the addition of the inhibitor. The inhibition reaction was biphasic at both 25° and 37°. An initial fast reaction was followed by a slower linear one. The rate of the fast reaction was dependent on the temperature and pH of the incubation medium and on the concentrations of both the inhibitor and dTMP synthetase except when either was present in a presumed excess.

The inhibitor acted directly on dTMP synthetase and was not an enzyme invalidating the assay for the latter. Two possible mechanisms for the action of the inhibitor were considered. It could either act catalytically, or it could act stoichiometrically to form a stable complex with dTMP synthetase. It was not possible to distinguish between these two mechanisms experimentally, the chief problems being the instability of the inhibitor and the absence of any means of identifying inhibited dTMP synthetase. Purification of the two proteins should eventually allow the mechanism of the interaction to be determined. The effect of pH on the interaction of dTMP synthetase and the inhibitor was analogucous to its effect on the

interaction of the pancreatic trypsin inhibitor with trypsin, and a stoichiometric mechanism is perhaps more likely for this reason.

The degree of inhibition of dTMP synthetase *in vivo* is unknown. It cannot be deduced from the activity found in cell-free extracts because when infected cells were sonicated at pH6 instead of at the usual pH of 7.5, the extracts contained uninhibited amounts of dTMP synthetase. Thus if the inhibitor functions *in vivo* the inhibition must be reversed by the violent conditions of cellular disruption, and the final activity of dTMP synthetase observed in cell-free extracts would then be due to a pH-dependent reassociation with the inhibitor. Although these observations also favour a reversible stoichiometric mechanism for the inhibition of dTMP synthetase, since the proposed inhibitor enzyme complex has not yet been dissociated *in vitro*, the possibility that the inhibitor and dTMP synthetase are brought together by cellular disruption cannot be excluded.

The physiological role of the inhibitor.

Assuming that the inhibition occurs *in vivo*, what is its physiological significance? Infection with phage SP8, which like phage Øe contains hydroxymethyluracil in its DNA, was found to cause some inhibition of dTMP synthetase, although it was less

effective in this respect than phage Øe. Since phage SP8 induced a dTTPase the possibility that phage Øe did likewise was investigated by studying the multiplication of phage Øe in a thymine-requiring host strain supplied with exogeneous thymidine. Multiplication was normal and no thymidine was incorporated into phage DNA. Since in this host the absence of dTMP synthetase is bypassed by the obligatory external supply of thymidine, the result implied that phage Øe induced a second block in thymine biosynthetsis. The induction of a dTTPase by phage Øe was confirmed by Dr. Roscoe.

In view of these results, what is the reason for the inhibition of dTMP synthetase during phage infection? In spite of the presence of a dTTPase the inhibition of dTMP synthetase could have a marginal role. Thymidylate synthetase is believed to be the main mechanism of thymine biosynthesis in B. subtilis although it is not the only source of dTMP. Its inhibition during phage infection would prevent the wasteful removal of dUMP, which is utilised by the dUMP hydroxymethylase for the biosynthesis of hydroxymethyluracil. In addition, the product of the action of both dTMP synthetase and the dTTPase is dTMP. In the absence of an inhibitor of dTMP synthetase dTMP would just accumulate in the phage-infected cell where it has no known role and in which it may even be inhibitory.

Conclusion

This work has provided an interesting example of the inhibition of an enzyme by a protein in circumstances which suggest that the inhibition could play a role in ensuring successful viral infection.

The inhibition may be one of an increasing number of examples in which the inactivation of an enzyme by a protein inhibitor is provoked by a sudden change in the metabolic balance of a cell.

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ABBREVIATIONS

Nucleotides and nucleosides

UMP, UDP, and UTP = uridine 5'-monophosphate, diphosphate and triphosphate respectively.

CDP, and CTP = cytidine 5'-diphosphate and triphosphate respectively.

dU, dUMP, and dUDP = deoxyuridine, deoxyuridine 5'-monophosphate and diphosphate respectively.

dCMP, dCDP, and dCTP = deoxycytidine 5'-monophosphate, diphosphate and triphosphate respectively.

dC, and dT = deoxycytidine and thymidine respectively.

dTMP, dTDP, and dTTP = thymidine 5'-monophosphate, diphosphate and triphosphate respectively.

dHUMP, dHUDP, and dHUTP = 5-hydroxymethyldeoxyuridine 5'-monophosphate, diphosphate and triphosphate respectively.

ATP = adenosine triphosphate.

Folic acid derivatives

H_4PteGlu = tetrahydropteroylglutamic acid.

H_4PteGlu$_3$ = tetrahydropteroyltriglutamic acid.

H_2PteGlu = dihydropteroylglutamic acid.

5,10-CH_2H_4PteGlu = 5,10-methylenetetrahydropteroylglutamic acid.

Others

DNA = Deoxyribonucleic acid.

RNA = Ribonucleic acid.

DMM = Davis minimal medium.

TCA	= Trichloracetic acid
p.f.u.	= plaque-forming units
Tris	= 2-amino-2-hydroxymethylpropane-1,3-diol
DEAE-cellulose	= o-(diethylaminoethyl)-cellulose
EDTA	= Ethylenediaminetetraacetate.
DPN	= Diphosphopyridine nucleotide

INTRODUCTION

INTRODUCTION

General Introduction

The study of bacteriophage infection has made important contributions to the fields of molecular biology and virology. The interrelationship of the bacteriophage and its host provides a comparatively simple system with great experimental flexibility. It has been used to investigate problems as diverse as the processes of DNA replication, the sequential induction of enzymes and its control, and the nature of mutation and the fine structure of a gene. In addition, the extensively studied bacteriophage systems provide useful models for the more complex problems of animal virus infection.

Lwoff (1957) defined a bacteriophage as "a strict parasite of bacteria possessing an infectious phase and which is multiplied in the form of its genetic material and which is sometimes pathogenic." Electron microscopy shows that most bacteriophages are tadpole-shaped with a hexagonal head attached to a tail (e.g. Kellenberger, 1961; Bradley, 1965). Exceptions are the minute phages ØX174, ØR and related phages and phages f2 and MS2 (e.g. Hall, Maclean and Tessman, 1959; Kay and Bradley, 1962; Davis, Strauss and Sinsheimer, 1961; Loeb and Zinder, 1961,) which have no apparent tail and the filamentous phages such as M13 (Salivar, Tzagoloff and Pratt, 1964). All bacteriophages are nucleoprotein

particles. The nucleic acid is usually double-stranded DNA (e.g. Sinsheimer, 1960) but may also be single-stranded DNA (Sinsheimer, 1959; Kay, 1962) or single-stranded RNA (Loeb and Zinder, 1961; Davis et al. 1961).

Bacteriophages multiply within a sensitive bacterial cell. The multiplication process of a virulent phage may be divided into the following stages: (Stent, 1963).

1. The bacteriophage adsorbs on to specific receptor sites on the bacterial cell wall.
2. The bacteriophage DNA is injected into the cell leaving the protein coat attached to the outside of the cell. The latter plays no futher part in the production of mature phage (Hershey and Chase, 1953).
3. The viral DNA dominates and reorganises the metabolism of the host cell such that it is redirected towards the biosynthesis of phage-induced enzymes, phage DNA and phage structural protein.
4. The bacterial cell lyses liberating mature phage progery.

Temperate phages have an an alternative to stage 3 in which they form a metastable symbiotic relationship with the host cell. The association may be perpetuated for many bacterial generations before the ultimate onset of phage multiplication and cellular lysis.

Bacteriophages of Bacillus subtilis

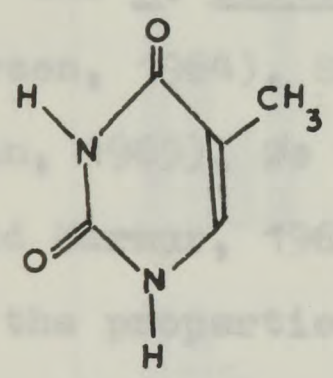
Until about 1960 most detailed research into the processes of bacteriophage infection was confined to phages of the Enterobacteriaceae and in particular to the T-series of coliphages. Bacteriophages of Bacillus subtilis were first isolated from soil by Romig and Brodetsky (1961). B. subtilis is a Gram-positive, aerobic sporing organism which can grow in a simple chemically-defined medium (Knight and Proom, 1950). Thus it is possible to isolate the various auxotrophic mutants which facilitate the investigation of various aspects of phage infection (e.g. Brabander and Romig, 1960). In addition, the discovery of Spizizen (1958) that B. subtilis DNA could be used to transform certain strains of B. subtilis permitted the genetic analysis of the organism. Therefore B. subtilis is a particularly suitable host in which to study phage infection.

At least fifty bacteriophages of B. subtilis are now known. They fall into a number of different categories which include a group of virulent phages whose DNA contains hydroxymethyluracil in place of thymine (Kallen, Simon and Marmur, 1962; Green, 1964; Okubo, Strauss and Stodolsky, 1964; Aposhian, 1965; Roscoe and Tucker, 1964; Pene and Marmur, 1964); a group of transducing phages whose DNA contains uracil in place of thymine (Takahashi and Marmur, 1963); a group of temperate phages (Tucker, 1964; Romig, 1962; Ikeda,

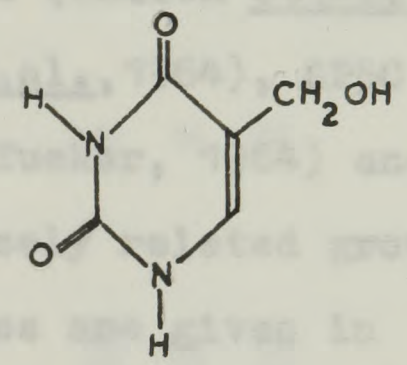
Saito, Miura, Takagi and Aoki, 1965); and a group of extremely defective phages which may be induced in every strain of B. subtilis so far examined (Seaman, Terzy and Marmur, 1964; Kiserling and Romig, 1964; Stickler, Kay and Tucker, 1965; Subbaish, Goldthwaite and Marmur, 1965).

Virulent bacteriophages whose DNA contains hydroxymethyluracil

This thesis is concerned with the group of B. subtilis bacteriophages whose DNA contains hydroxymethyluracil in place of thymine. The structures of the two pyrimidines are compared below.



Thymine



Hydroxymethyluracil

An unusual base was first detected in the DNA of the virulent T-even coliphages, in which cytosine is replaced by hydroxymethylcytosine (Wyatt and Cohen, 1953). The unusual base is most useful for investigating the phage infection process since it both distinguishes the phage DNA from the host-cell DNA and facilitates the identification of a number of phage-induced enzymes. The T-even coliphages have therefore been extensively used to study the metabolic changes brought about by phage infection, and the discovery that a group of B. subtilis

phages also contained an unusual pyrimidine base stimulated a considerable amount of research on the new system. In some respects B. subtilis is a better host to use than E. coli because actinomycin D will penetrate the former without treatment and can therefore be used to inhibit DNA-dependent RNA synthesis (Levinthal, Keynan and Higa, 1962; Levinthal, Fan, Higa and Zimmerman, 1963). In addition, phage DNA may be used to transfect competent strains of B. subtilis (Green, 1964; Romig, 1962).

The B. subtilis phages SP8 (Kallen et al., 1962) SP82 (Green, 1964), SP01 (Okubo et al., 1964), SP5C (Aposhian, 1965), Øe (Roscoe and Tucker, 1964) and 2C (Pene and Marmur, 1964) form a closely related group. Some of the properties of the phages are given in Table 1. They are slightly larger than the comparable group of T-even phages but have the same tadpole shape. The DNA of phage SP82 has the same molecular weight of 130×10^6 as the DNA of a T-even phage (Green, 1964; Cairns, 1961; Kleinschmidt, Lang, Jacherts and Zahn, 1962). An unusual property of the DNA of all these B. subtilis phages is its denaturation into two strands of different buoyant density. Marmur and Greenspan (1963) reported that only the heavy strand forms hybrids with the RNA formed in the infected host.

The B. subtilis phages appear to differ somewhat

Table 1. Properties of the phages of B. subtilis whose DNA contains hydroxymethyluracil

Phage	DNA				Dimensions		References
	T_m	% G+C	Buoyant density g/cm³	Number of strands when denatured	Head diameter mμ	Tail length mμ	
Øe	76.5°	40	1.743	2	90	180	Roscoe & Tucker (1966
SP8	77.5°	43	1.743	2	100	200	Marmur & Cordes (1963) Davison (1963) Kallen et al.(1962)
SP01		44.9	1.740	2	100	200	Okubo et al. (1964)
SP82	75°	43	1.742	2			Green (1964)
2C	78°	43	1.741	2			Pene & Marmur (1964)

from the T-even coliphages in their effect on the host DNA, and on RNA synthesis. Neither phage Øe nor phage SP8 induces the degradation of the host-cell DNA (Marmur, Greenspan, Palecek, Kahan, Levine and Mandel, 1963; Roscoe, 1965), although this occurs during T-even infection of E. coli. Two different effects of phage infection on the net RNA synthesis in the host cell have been reported. Phage Øe, like phage T2, causes a cessation of net RNA synthesis (Roscoe, 1965) whereas phages SP01 and SP8 do not (Marmur et al., 1963; Levinthal, Hosoda and Shub, 1966). It has yet to be determined whether this is a phage-specific difference or due to the use of different host strains of B. subtilis.

Since the infected B. subtilis cell contains two types of DNA available for transcription, the messenger RNA formed during infection has been investigated. Marmur et al., (1963) reported that some RNA specific for the host DNA is synthesised during phage SP8 infection, and Gage, Wilson and Geiduschek (1967) obtained similar results with phage SP01. In addition, Levinthal et al., (1966) found that during phage SP01 infection host protein synthesis continued at a level of about 10%. Therefore the B. subtilis phages, unlike the T-even coliphages, may require some host cell functions.

Enzyme changes induced by *B. subtilis* phages

By analogy with the T-even coliphages it would be expected that at least two different classes of phage-induced enzymes would be detected in the infected cell. They are firstly, enzymes involved in the biosynthesis of hydroxymethyluracil and secondly, enzymes involved in preventing the accumulation of thymidine triphosphate. The latter class of enzymes are required because DNA polymerases are non-specific in their requirement for nucleoside triphosphates (Okazaki and Kornberg, 1964). If thymidine triphosphate accumulated in the infected cell it would probably be incorporated into phage DNA.

The biosynthesis of pyrimidine nucleotides in the host cell is shown in Fig. 1. which assumes that *B. subtilis* resembles *E. coli* in this respect. A number of phage-induced changes are also indicated. It must be emphasised that not all these changes are necessarily induced by every phage whose DNA contains hydroxymethyluracil.

The biosynthesis of hydroxymethyluracil is satisfactorily accounted for by the presence in infected cells of the enzyme dUMP hydroxymethylase (Kahan, Kahan and Riddle, 1964; Roscoe and Tucker, 1964, 1966). It converts dUMP to dHUMP and has a catalytic requirement for H_4PteGlu which presumably acts as a carrier for the C_1 unit. Although the enzyme catalyses a reaction which

Figure 1.

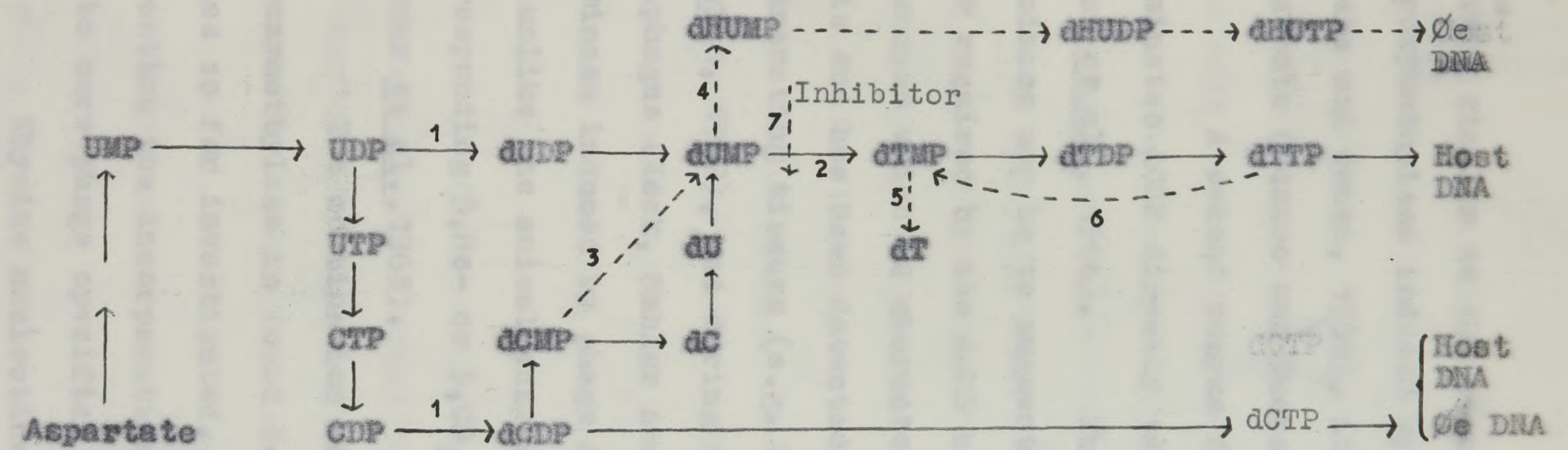


Fig. 1. Pyrimidine biosynthesis in normal (——→) and phage-infected (- - - - →) B. subtilis. (from Davidson, 1965; and Dilworth-Cannon & Breitman, 1967).

The following enzymes are of particular interest: (1) Ribonucleotide reductase (Larsson & Reichard, 1966); (2) dTMP synthetase (Wahba & Freidkin, 1962); (3) dCMP deaminase and (4) dUMP hydroxymethylase (Kahan, Kahan & Riddle, 1964; Roscoe & Tucker, 1966); (5) dTMPase (Aposhian, 1965); (6) dTTPase (Kahan, Kahan & Riddle, 1964); (7) Is an inhibitor of dTMP synthetase (Roscoe & Tucker, 1966).

is very similar to the reaction catalysed by the dCMP hydroxymethylase induced by the T-even coliphages (Flaks and Cohen, 1959), it cannot utilise dCMP as a substrate (Roscoe and Tucker, 1966).

A second enzyme found in phage-infected cells deaminates dCMP directly to dUMP (Roscoe and Tucker, 1966; Kahan et al., 1964). The function of this dCMP deaminase may be to augment the intracellular supply of dUMP required by the dUMP hydroxymethylase. It is an enzyme which is characteristic of rapidly proliferating cells and has been detected in animal embryonic and regenerating tissues (e.g. Maley and Maley, 1964; Scarano et al., 1962), and during infection by the T-even coliphages (Keck, Mahler and Fraser, 1960). The dCMP deaminase induced by phage SP8 is specific for dCMP and unlike the animal enzymes does not attack the corresponding 5,Me- or 5,CH_2OH-substituted nucleotides (Marmur et al.,1963).

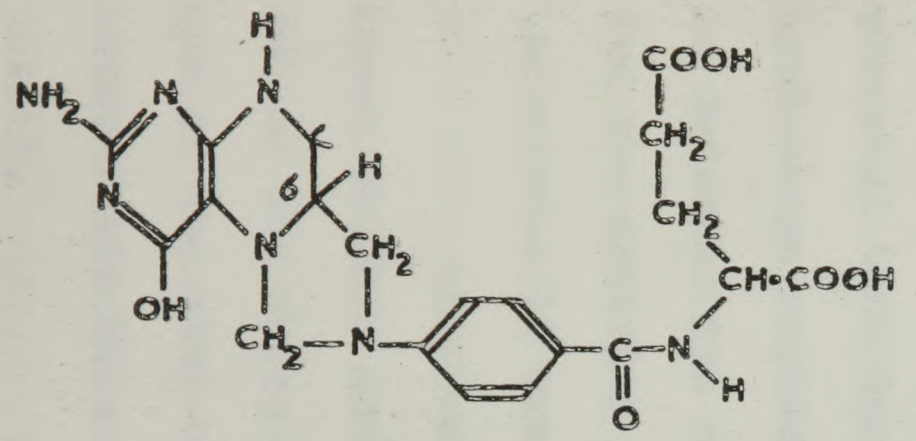
The combination of dCMP deaminase and dUMP hydroxymethylase is found during infection by all the phages so far investigated. However, the mechanisms for preventing the incorporation of thymine into phage DNA may be more phage specific.

Thymine nucleotides may arise intracellularly in a number of different ways. The only firmly established pathway of de novo thymine biosynthesis

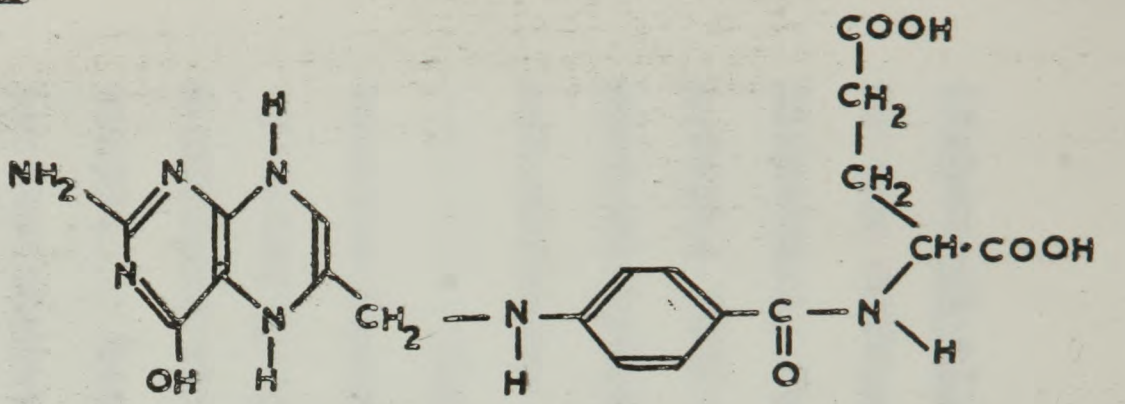
involves the conversion of dUMP to dTMP (Wahba and Friedkin, 1962; Greenberg, Nath and Humphreys, 1961; Blakley and McDougall, 1962). This methylation reaction is catalysed by thymidylate synthetase and involves H_4PteGlu which acts both as a donor of the C_1 unit and as the reducing agent. The reaction is shown in Fig. 2. Recently some genetic and physiological evidence has suggested that in B. subtilis there may be a second pathway of de novo thymine biosynthesis (Wilson, Farmer and Rothman, 1966; Anagnostopoulos and Schneider-Champagne, 1966). The biochemistry of this pathway is unknown although Wilson et al., (1966) suggest that it may involve a methylation of uracil deoxyribonucleoside or a derivative with H_4PteGlu$_3$ as cofactor. Therefore this pathway is a probable source of thymidine or a phosphorylated derivative.

In addition to the two methylation reactions, thymidylate is also formed by the degradation of DNA to 5'-nucleoside monophosphates, and by the deoxythymidine kinase salvage mechanism described by Okazaki and Kornberg (1964 a and b). In view of the number of ways in which thymine nucleotides may arise in the cell the most effective way to prevent the incorporation of thymine into phage DNA would be to destroy thymidine triphosphate. Phage SP8 induces a dTTPase which hydrolyses the terminal pyrophosphate group from dTTP to produce dTMP

Figure 2

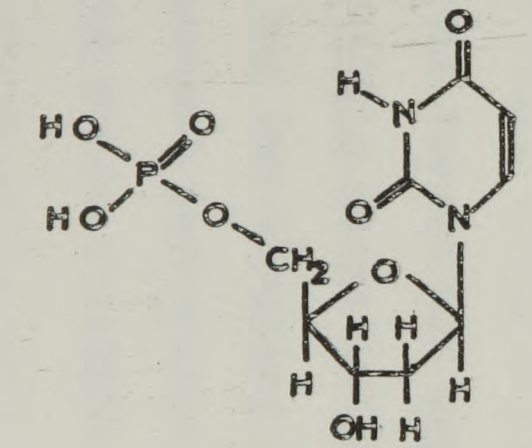


5,10-CH_2-H_4PteGlu

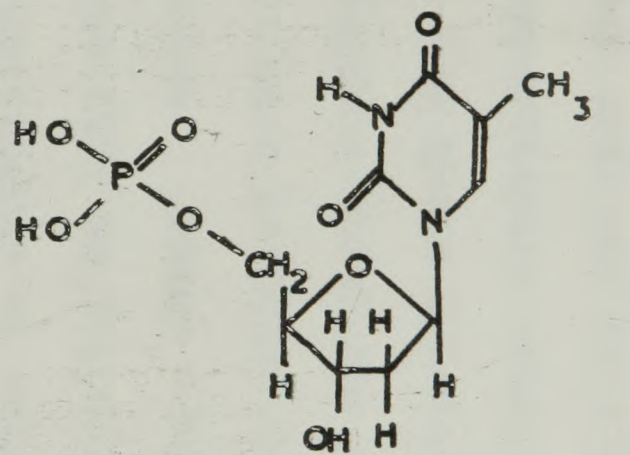


H_2PteGlu

Mg^{++}



dUMP



dTMP

Fig. 2. The reaction catalysed by dTMP synthetase

The hydrogen atom at the 6 position of H_4PteGlu is incorporated into the methyl group of dTMP and H_2PteGlu is formed (Pastore and Friedkin, 1962 ; Blakley, Ramasastri and M^{c}Dougall, 1963).

(Kahan et al., 1964). This reaction is analogous to the dCTPase induced by the T-even coliphages (Kornberg, Zimmerman, Kornberg and Josse, 1959) except that the product dCMP in the latter case may be utilised to produce hydroxymethyldeoxycytidylic acid, whereas dTMP has no known function in the phage SP8-infected cell.

Two other mechanisms which may also block thymine biosynthesis have been detected during infection by other B. subtilis phages. Phage SP50 induces a dTMPase which hydrolyses dTMP to thymidine (Aposhian, 1965). However the significance of this enzyme is confused by the observation that it also increases in activity after infection of B. subtilis by phage whose DNA contains thymine (Aposhian, 1965). The second mechanism is most interesting. Roscoe and Tucker (1964, 1966) observed that during phage Øe infection the enzyme dTMP synthetase rapidly disappeared. No activity could be detected ten minutes after the addition of phage Øe and the activity was not restored by dialysis of the infected-cell extracts.

When work on this thesis was begun it was not known whether each B. subtilis phage induced more than one of the above mechanisms for blocking thymine biosynthesis. One of the aims of the present investigation was to investigate this aspect of phage infection in more detail.

The present investigation

The infection of *B. subtilis* by phage Øe has been investigated. This phage was chosen because of the unusual observation of Roscoe and Tucker (1964, 1966) that dTMP synthetase rapidly disappeared during infection. The most frequently encountered mechanisms for controlling the activities of enzymes in bacteria are feed-back inhibition and the repression or induction of enzyme synthesis (Jacob and Monod, 1961; Ames and Martin, 1964; Monod, Wyman and Changeux, 1965; Cline and Bock, 1966; Stadtman, 1966). Neither of these possibilities appeared to explain the disappearance of dTMP synthetase. If the enzyme were affected by feed-back inhibition it should have been reactivated by dialysis, and if the disappearance were due to a cessation of enzyme synthesis dTMP synthetase would have to be metabolically a very unstable enzyme. There was no evidence for the latter hypothesis. Therefore the operation of an unusual method of control was indicated. Reports of other enzymes being rapidly inhibited are extremely rare. However, as the following examples illustrate, the phenomenon has been observed both during infection by the coliphages and after the cessation of the synthesis of inducible or repressible enzymes in bacteria and yeasts.

During the infection of *E. coli* by the T-even

phages two enzymes are inactivated. In both cases the inactivation is prevented by the presence of chloramphenicol during the infection. Ortiz, August, Watanabe, Kaye and Hurwitz (1965) found that ATP: polynucleotide adenylytransferase (August, Ortiz and Hurwitz, 1962) was rapidly inhibited and that the inhibition was not reversed either by dialysis or by partial purification of the enzyme. Sköld and Buchanan (1964) found that DNA-dependent RNA polymerase was also inhibited but in this case the inhibition was reversed by a partial purification of the enzyme (Furth and Pizer, 1966). The nature of the inhibitors of these two enzymes and their roles during phage infection are unknown. The origin of the inhibitor of DNA-dependent RNA polymerase is obscure, since T2 ghosts can also cause inhibition of the enzyme (Khesin, Shemyakin, Gorlenko, Bogdanova and Afanas'eva, 1962), and an inhibitor may also be isolated from uninfected E. coli (Furth and Pizer, 1966; Oleson, 1966).

Enzyme inactivations accompanying the cessation of enzyme synthesis have been reported in a number of systems (e.g. Mandelstam, 1954; Robertson and Halvorsen, 1957; Witt, Kronau and Holzer, 1966; Ferguson, Boll and Holzer, 1967). In two examples of this phenomenon protein inhibitors of the enzymes have been identified. Bechet and Wiame (1965) detected the

appearance of a protein which inhibited the activity of the existing ornithine transcarbamylase in Saccharomyces cerevisiae when the synthesis of the enzyme was repressed by the addition of arginine to the medium. Similarly Mecke, Wulff, Liess and Holzer (1966) observed a protein which inactivated glutamine synthetase activity in E. coli after the addition of $(NH_4)^+$ ions to the medium to repress the synthesis of the enzyme.

The object of the investigation

It was decided to investigate the phage-induced disappearance of dTMP synthetase activity. The phenomenon was of interest both because of its possible role during phage infection and because enzyme inactivation mechanisms are an unusual aspect of the problem of metabolic control of enzyme activity.

It was hoped to determine how dTMP synthetase was inactivated and to assess the physiological significance of the process.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism and phage

Bacteria. The following bacterial strains were used:- Bacillus subtilis 3610, a Marburg strain; B. subtilis 3/13, a tyrosine auxotroph derived from B. subtilis 3610 by ultraviolet irradiation; B. subtilis 168, the indole-requiring strain described by Spizizen (1958); B. subtilis 168T⁻ was a gift from Dr. Farmer and was the thymine-requiring double mutant derived from B. subtilis 168 described by Farmer and Rothman (1965). Stocks were maintained on nutrient agar slopes which were supplemented with $3 \cdot 10^{-4}$M thymine in the case of B. subtilis 168T⁻, and stored at 4°.

Phage. Phage Øe was isolated from soil (Roscoe, 1962). Stocks were prepared by lysis of B. subtilis 3610 grown in litre batches in nutrient broth, and the phage was purified by differential centrifugation (Roscoe and Tucker, 1966). Phage SP8* was a gift from Dr. Romig and was the phage SP8 described by Romig and Brodetsky (1961) but with an extended host range. A stock was obtained from a nutrient broth lysate of B. subtilis 168 by the method used to prepare and purify phage Øe. All phage stocks were suspended over a crystal of thymol in phage phosphate buffer. This contained per litre: 7g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl,

246 mg $MgSO_4.7H_2O$, 12 mg anhydrous $CaCl_2$ and 10 mg gelatin. The pH was 7.

Liquid media

All growth media were sterilised by autoclaving at 15 lb/sq in for 15 min. All supplements were added after separate sterilisation.

Nutrient broth (Lennox, 1955) contained per litre: 10g tryptone (Oxoid), 5g yeast extract (Difco) and 10g NaCl. The pH was 7.2.

Minimal Medium, DMM (Davis and Mingioli, 1950). Double strength medium was used and contained per litre: 14g K_2HPO_4, 6g KH_2PO_4, 1g sodium citrate.$2H_2O$, 2g $(NH_4)_2SO_4$ and 200 mg $MgSO_4.7H_2O$.

Supplemented DMM was DMM supplemented with ferric citrate (40μg per ml. of both $FeCl_3.6H_2O$ and sodium citrate.$2H_2O$) and with $4 \cdot 10^{-2}$M D-glucose.

Sulphate-free DMM was DMM containing 0.8g NH_4Cl in place of $(NH_4)_2SO_4$ and 165mg $MgCl_2.6H_2O$ in place of $MgSO_4.7H_2O$. It supported the normal growth of B. subtilis 3610 when supplemented with $1.25 \cdot 10^{-4}$M Na_2SO_4.

Methods used to set up bacterial cultures

Bacterial growth was followed by estimating turbidity with an EEL photoelectric colorimeter (Evans Electroselenuim Ltd., Halstead, Essex). The

viable count of a culture was determined by spreading known volumes, after dilution in the appropriate growth medium, on nutrient agar plates and counting colonies after incubation at 30° overnight. A standard curve relating visble count to EEL reading was thus prepared. All growth in liquid culture was at 37°. Two methods were used to set up cultures.

Method 1. (Roscoe and Tucker, 1966). B. subtilis 3610 was grown up overnight in supplemented DMM containing 0.01% (w/v) acid-hydrolysed casein (Difco); after harvesting and washing in DMM the organisms were resuspended in approximately four times the original culture volume, at an EEL reading of 0.5, using fresh supplemented DMM.

Cultures of B. subtilis 3/13 were all supplemented with $5 \cdot 10^{-4}$M D,L-tyrosine. When tyrosine-starved B. subtilis 3/13 were required the bacteria were collected by centrifugation, washed with DMM and resuspended in a volume of warm (37°) supplemented DMM (omitting tyrosine) equal to the original volume.

B. subtilis 168 grew poorly under the conditions described above. Overnight cultures were grown on nutrient broth supplemented with $5 \cdot 10^{-3}$M D-glucose. After harvesting and washing in DMM the organisms were

suspended (at EEL = 0.5) in fresh supplemented DMM containing 0.05% acid-hydrolysed casein (Difco) and $5 \cdot 10^{-4}$M D,L-tryptophan.

B. subtilis 168T⁻ was grown exactly as described for B. subtilis 168 except that $2 \cdot 10^{-4}$M thymidine was added to the overnight culture and $1 \cdot 10^{-4}$M thymidine was added to the culture in supplemented DMM.

Method 2. In later experiments with B. subtilis 3610 Method 1 was simplified as follows. The overnight culture was grown on supplemented DMM containing 0.1% (w/v) acid-hydrolysed casein (Difco) and diluted 10 fold into identical fresh medium. The initial EEL reading was 0.5 as in Method 1, the additional presence of acid-hydrolysed casein shortened the lag phase of the culture but did not affect the growth rate.

Aeration of cultures. Bacterial cultures were aerated in 3 different ways. In the first method 10ml volumes in T-shaped tubes were rocked in a water bath at 36 oscillations per min. In the second method 50-400 ml volumes in corresponding 250-2000 ml conical flasks were shaken on a gyrotary platform at 220 revolutions per min. In the third method 1 litre volumes in 5l flasks were swirled on an apparatus of the type described by Mitchell (1949).

It was usual to use the first method to study

phage single-step growth curves, the second method to assay for enzymes in cell-free extracts, and the third method to isolate enzymes on a large scale.

Phage infection and multiplication

Bacterial cultures were always infected with phage in early log phase at $3 \cdot 10^8$ viable cells per ml. (EEL = 2.0) at a multiplicity of mixing of 5 p.f.u. per viable cell. Phage infections were followed either by the lysis of the infected culture, or by determining the bacterial viable count just before, and 10 min after the addition of phage, or by single-step growth curves of infected cultures diluted 5 min after the addition of phage (Ellis and Delbrück, 1939). The choice of method or combination of methods depended on the experiment.

Phage assay. Phage suspensions or phage-infected cultures were assayed by the agar over-layer technique (Adams, 1959) using 10^8 spores of B. subtilis 3610 per Petri dish to give the indicator lawn for phage Øe. Phage SP8 was assayed using spores of B. subtilis 168. All incubations were overnight at 37°. The spores of B. subtilis 3610 and B. subtilis 168 were grown in potato extract (Spizizen, 1958), collected and washed

3 times with sterile H_2O by centrifugation, and stored at 4° as a concentrated suspension in H_2O.

The preparation of bacterial extracts for measurement of enzyme activities (Roscoe and Tucker, 1966).

Up to 6 parallel 100-200 ml. volumes of a culture of B. subtilis in 1 litre conical flasks were aerated until the EEL reading was 2.0. The cultures were then infected with phage. At various times after infection they were rapidly chilled by pouring onto an equal volume of frozen DMM, collected by centrifugation, washed with buffer B and resuspended in 5 ml. buffer A per 100 ml. of original culture. The resuspended cells were disrupted at 25 kc per sec. for 4 min. at 0° using a Mullard Ultrasonic Generator type E 75090B or using the Ultrasonic Power Unit model 3000, made by Measuring and Scientific Equipment Ltd., London, S.W.1. Sonicated extracts were clarified by centrifugation at 10 000x g for 5 min. and 100 000 x g for 60 min. All operations subsequent to chilling the culture were conducted at 4°. The protein concentration of the extracts was approx. 4mg per ml. Extracts of uninfected bacteria were prepared by the same method.

The large scale preparation of extracts from infected cells

The initial cell-free extract from which the inhibitor of dTMP synthetase was isolated was prepared

as follows. Using Method 2 to set up the cultures, 6l of B. subtilis 3610 were infected with phage Øe. The infected cultures were chilled with frozen DMM 25 min. after the addition of phage, harvested by centrifugation, washed with buffer B and resuspended in 9 ml. buffer A per litre of culture. The resuspended cells were usually stored at -15° before disruption in 15 ml. batches by sonication at 0° for 5 min. The sonicated extract was clarified by centrifugation at 100 000 xg for 60 min. and was then ready for fractionation with $(NH_4)_2SO_4$.

The large scale preparation of extracts from uninfected cells

The initial cell-free extracts from which dTMP synthetase was isolated was prepared from B. subtilis 3610 grown on either minimal medium or nutrient broth.

Preparation of the $(NH_4)_2SO_4$ fraction used in experiments described in Chapter II

Using Method 2 to set up the cultures, 6l of B. subtilis 3610 were grown to a density of $2 \cdot 10^9$ viable cells per ml. (EEL = 4), centrifuged, washed with buffer B and resuspended for disruption in 18 ml. buffer A per litre of culture. The resuspended cells were disrupted by sonication in 15 ml. batches at 0° for 5 min. The sonicated extracts were centrifuged for 5 min. at 10 000 xg, stirred with 2.5 ml. of 2.5% (w/v) streptomycin

sulphate solution per 15 ml. of extract for 15 min. at 4^{o}, and finally centrifuged at 100 000 xg for 60 min. The supernatant was then ready for fractionation with $(NH_4)_2SO_4$.

Preparation of the initial cell-free extract for the purification of dTMP synthetase described in Chapter III

An overnight culture of 50 ml. of B. subtilis 3610 in nutrient broth containing $5 \cdot 10^{-3}$M D-glucose was tipped into 950 ml. of nutrient broth containing $5 \cdot 10^{-2}$M D-glucose. Aeration was continued until the EEL reading was 5.5. The bacteria were collected by centrifugation, washed with buffer B and resuspended in 25 ml. buffer A per litre of culture. Thereafter the procedure was as described above. It was usual to grow 12l of B. subtilis 3610 at a time.

The purification of phage Øe for DNA isolation

Cultures (2.5l) of B. subtilis 168T$^-$ were infected with phage Øe and aerated until lysis occurred. The lysate was centrifuged at 2 000 xg for 15 min. and the supernatant then centrifuged at 23 000 xg for 60 min. The phage pellets were resuspended in approx. 40 ml. of phage phosphate buffer and incubated at 37^{o} for 1h with 1 mg. per ml. each of lysozyme, DNAse I and RNAse. The suspension was then centrifuged at 10 000 xg for 5 min. and the supernatant was diluted to 1l with phage phosphate

buffer and centrifuged at 23 000 xg for 60 min. The phage pellets were resuspended in 9 ml. 0.1M sodium phosphate buffer pH 6.7 and finally centrifuged twice at 10 000 xg for 5 min. The overall recovery of phage in the final supernatant was 45%.

The isolation of phage Øe DNA.

DNA was prepared by the method of Mandel and Hershey (1960). The buffered phage suspension (9 ml.) was chilled and rocked gently with an equal volume of chilled buffer-saturated phenol in the presence of 0.1 ml of 25% dodecyl sulphate. The aqueous layer was separated from the phenol layer and the extraction was repeated. The aqueous layer was then extracted 4 times with an equal volume of chilled diethyl ether. DNA was precipitated 4 times from the aqueous layer by adding 2 volumes of chilled ethanol. The DNA precipitate was finally redissolved in 7 ml. H_2O (approx. 0.5 mg DNA per ml.) containing 0.1M $MgCl_2$.

Enzymic hydrolysis of DNA (Roscoe, 1965)

The DNA solution was incubated at 37° with DNAse I (concn. 3µg per ml.). As acid groups were released the pH was maintained at 7 by the addition of 0.1 N NaOH. After 30 min. the pH was increased to 9.1 and snake venom phosphodiesterase (50µg per ml.) was

added. Acid groups were neutralised at the new pH as before until the reaction was complete (75 min.). The hydrolysate was deproteinised with chloroform/iso-amyl alcohol (24:1, v/v) (Marmur, 1961); the volume was reduced by half by evaporation in a stream of air at 25° and the nucleotides were separated by paper chromatography.

Location of nucleotides on paper

Ultraviolet-absorbing spots were located on paper chromatograms by making contact prints in ultraviolet light and tracing the spots from the prints back onto the papers (Markham and Smith, 1949).

Radioactive materials and methods

All radioactive materials were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. In all cases counts of radioactive material were taken over a period of time such that the percentage error was less than 5%.

Counting TCA-insoluble precipitates for ^{35}S

The precipitate was filtered on a Millipore membrane filter, type HA. (V.A. Howe, 46 Pembridge Road, London W.11.). The filter was washed twice with 5 ml. of 5% TCA, twice with 5 ml. of H_2O and was then transfered to an aluminium planchet moistened with 2 drops of 1% Cetavlon (cetyl trimethyl ammonium bromide). The

planchet was heated under a lamp until just dry and then placed inside a Panax lead castle (Panax Equipment Ltd., Mitcham, Surrey). It was counted by a thin end mica window Geiger-Müller tube type EHM25 (General Electric Co., England) and impulses were registered on an EKCO Automatic Scaler Type N530D (EKCO Electronics Ltd., England).

Preparation of (^{14}C) HCHO for use in the assay for the dUMP hydroxymethylase

(^{14}C) HCHO was obtained as paraformaldehyde and was depolymerised by maintaining at 110° with water in a sealed tube for 2 days. It was then mixed quantitatively with standardised unlabelled HCHO to give a solution which contained 10 μmoles HCHO per ml. at a specific activity of 1 μCurie per μmole.

Enzyme assays

1. dUMP hydroxymethylase

A modification of the assay method described by Roscoe and Tucker (1966) was used. The reaction mixture contained 0.5 μmoles (^{14}C)HCHO; 0.5 μmoles dUMP; 0.1 μmole H_4PteGlu; 25 μmoles Tris buffer A; 5 μmoles 2-mercaptoethanol; extract and water to a final volume

of 0.5 ml. After 45 min incubation at 37° 0.1 ml of $3 \cdot 10^{-2}$M hydroxylamine hydrochloride and 0.1 ml of bovine serum albumin solution (10 mg per ml) were added and the tube was placed in boiling water in a fume cupboard for 2 min. This procedure drove off the excess HCHO and precipitated the protein. The coagulated protein was removed by centrifugation and a portion of the supernatant fluid (0.15 ml) was applied to water-washed Whatman No.1. paper. Control incubations omitting dUMP were similarly treated. All incubations were set up in duplicate.

The chromatogram was developed by the ascending technique running internal dUMP and dTMP markers with each sample. The solvent was prepared by adding 600g $(NH_4)_2SO_4$ to 1l 0.1M potassium phosphate buffer pH 6.8. The solution was filtered and 20 ml n-propanol added to the filtrate (Wahba and Friedkin, 1962a). The position of the radioactive dHUMP was approximately the same as that of dUMP and adjacent to the dTMP. Strips of the chromatogram were counted in ½in sections in duplicate using a Chromatogram Scanner type 2029. (Isotope Developments Ltd., Beenham, Reading, Berkshire). No counts above background were found in the dHUMP region of the controls. The specific activity of the (^{14}C)HCHO was determined by counting the dHUMP obtained when all the HCHO was utilised in the presence of excess dUMP

and a highly active enzyme fraction. The enzyme activity was calculated as μmoles dHUMP formed (or μmoles HCHO fixed) per h at 37°. The assay was linear with time and protein concentration provided not more than 30% conversion of (^{14}C) HCHO to dHUMP occurred. The maximum specific activity of the dUMP hydroxymethylase in infected extracts was 7 fold higher than previously reported (Roscoe and Tucker, 1966). This may be due to a combination of different conditions of growth, infection and disruption of B. subtilis 3610 and modifications in the assay procedure.

2. dTMP synthetase

The oxidation of H_4PteGlu was followed spectrophotometrically as described by Wahba and Friedkin (1961, 1962). Two cuvettes each contained the following; 25 μmoles $MgCl_2$; 50 μmoles Tris buffer A; 100 μmoles 2-mercaptoethanol; 2 μmoles HCHO; 1.0 μmole EDTA; 0.2 μmoles H_4PteGlu; extracts and water to give a final volume of 0.9 ml. To start the reaction 0.1 μmole dUMP was added to one cuvette. The increase in absorbance at 340 mμ at 25° was followed against the blank cuvette using a Cary recording spectrophotometer, model 14, with fine slide-wire attachment (Applied Physics Corporation, Monrovia, California, U.S.A.) or a Zeiss spectrophotometer, model M4 QII (Carl Zeiss, Germany) coupled to a Gilford

model 2000 Multiple Sample Absorbance Recorder (Gilford Laboratories Incorporated, Oberlin 1, Ohio, U.S.A.) with the full scale deflection set for 0.1 optical density units.

This assay was reported to be suitable for the dTMP synthetase of E. coli only after some initial purification (Wahba and Friedkin, 1962a). With the crude extracts of B. subtilis some precipitation in the cuvettes occurred if the protein concentration chosen for the assay was too high. Precipitation was manifested by large changes in slit width or large increases in the absorption of the blank cuvette, depending on the spectrophotometric system used. However the assay was linear with protein provided no precipitation occurred, and it was also linear with time for at least 15 min.

When purified dTMP synthetase was assayed the precipitation problem vanished. The assay was linear with time and with protein concentration provided the increase in optical density units was less than 0.8 per h. 1 unit of dTMP synthetase oxidised 1 μmole H_4PteGlu per h at 25°.

Polyacrylamide gel electrophoresis

The method employed was essentially the 'simplified' method of Clarke (1964) in which a common Tris/glycine buffer (pH 8.1) was used in both electrode compartments and in the polyacrylamide gel, and in which the sample was applied to the top of the polymerised gel in

a 20% sucrose solution. The method was modified by the addition of riboflavin to the gel mix instead of ammonium persulphate so that the polymerisation was photocatalysed (Davis, 1964). The disc electrophoresis apparatus was supplied by Shandon Scientific Company Ltd., 65 Pound Lane, Willesden, London N.W.10.

The following solutions in 100 ml were stored at 4°. Solution A contained 2.9g glycine, 0.6g Tris, 0.23 ml N, N, N', N'-tetramethylethylenediamine (TEMED). Solution B contained 40 g sucrose. Solution C contained 25g acrylamide, 0.74g N, N'-methylenebisacrylamide (BIS). Solution D contained 4mg riboflavin. The gel was prepared by mixing 2 ml A, 8 ml B, 4 ml C and 2 ml D and pipetting 1.1 ml of the mixture into 8 stoppered electrophoresis tubes. When the gel had polymerised the tubes were inserted in the electrophoresis apparatus, the sample was applied to the top of the gel and the electrophoresis conducted at room temperature as described in the text. The gels were stained with amido black and destained by electrophoresis in 7% (v/v) acetic acid according to the instructions provided with the Shandon apparatus. Completed gels were stored under water at room temperature.

Buffer Solutions

Tris/HCl buffers were titrated to the appropriate pH

with 11N HCl. Buffer A was 10^{-1}M Tris containing 10^{-2}M 2-mercaptoethanol and 10^{-4}M EDTA (pH 7.5). Buffer B was $5 \cdot 10^{-2}$M Tris containing 10^{-2}M 2-mercaptoethanol and 10^{-4}M EDTA (pH 7.5). Buffer C was the same composition as Buffer B (pH 8.4). Buffer D was Buffer B containing 10% (v/v) A.R. glycerol which was found to stabilise both dTMP synthetase and its inhibitor to storage at 4°.

Tris acid maleate/NaOH buffers ($5 \cdot 10^{-2}$M) were made up as described by Gomori (1948).

Materials

All chemicals unless otherwise stated were analytical reagent grade where possible (British Drug Houses Ltd., Poole, Dorset). Alumina was Griffin polishing alumina Grade 5/20 (Griffin and George Ltd.,). Chloramphenicol was purchased from Parke, Davis and Co., Hounslow, Middlesex. 'Enzyme-grade' $(NH_4)_2SO_4$ was obtained from Mann Research Laboratories Inc., New York, or was prepared by treating A.R. $(NH_4)_2SO_4$ with Chelex 100 (A.R. chelating resin, Calbiochem). Purine and pyrimidine derivatives and Aquacide I were purchased from Calbiochem (California Foundation for Biochemical Research, Los Angeles 63, California, U.S.A.). Streptomycin sulphate was obtained from Glaxo Laboratories Ltd., Greenford, Essex. Tetrahydrofolic acid (H_4PteGlu) was prepared by the reduction of folic acid by the method

of Jones et al.(1961) and was stored at -15° under hydrogen in the presence of 10^{-3}M 2-mercaptoethanol. Visking dialysis tubing was washed before use according to the procedure of Wahba and Friedkin (1962).

Enzymes DNAse I and Snake-venom diesterase were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Crystallised egg white lysozyme, RNAse and trypsin (salt-free) were obtained from Armour Pharmaceutical Company Ltd., England. Soybean trypsin inhibitor (3x crystallised) was obtained from Sigma Chemical Company, U.S.A.

Column chromatography and gel filtration

DEAE-cellulose (DE50) was obtained from L.Light and Co. Ltd., and was washed and equilibrated with buffer C according to the procedure given by Peterson and Sober (1962). All Sephadex powders were obtained from Pharmacia, Uppsala, Sweden, and were swollen by the method recommended in the accompanying technical literature.

Chemical Estimations

Protein. Soluble protein was measured by the method of Lowry, Rosebrough, Farr and Randall (1951) with a standard of crystalline bovine serum albumin.

Deoxyribonucleic acid. The DNA content of TCA extracts or of solutions of purified DNA was measured by the

method of Burton (1956). The standard curve was prepared from 2-D-deoxyribose (Calbiochem). To extract the DNA from TCA precipitates of bacterial cultures the precipitates were collected by centrifugation and washed with 5 ml cold 5% TCA. The pellets were heated at 90° for 15 min in 2 ml 5% TCA. After centrifugation, portions of the supernatant fluids were assayed for DNA.

RESULTS

RESULTS

CHAPTER I. The cause and possible physiological role of the inhibition of dTMP synthetase

Introduction

At the beginning of this work a number of explanations for the nature and physiological role of the inhibition of dTMP synthetase during phage infection were considered.

The possible causes of the inhibition were as follows:

1. That phage Øe injected an inhibitor of dTMP synthetase into the host cell.
2. That dTMP synthetase was a metabolically unstable enzyme with a high turnover rate.
3. That phage Øe infection resulted in the synthesis of an inhibitor, or in the synthesis of a factor interfering with the assay for dTMP synthetase.

This chapter describes experiments which eliminated the first two possibilities by showing that the disappearance of dTMP synthetase was prevented by inhibiting protein synthesis during phage infection. The identification of an inhibitor of dTMP synthetase confirmed the third possibility.

The apparent physiological role of the inhibition was to prevent the incorporation of thymine nucleotides

into phage Øe DNA. One method of assessing the importance of such a role was to study the multiplication of phage Øe in a thymine-requiring host in which the absence of an intracellular supply of thymine nucleotides could be bypassed by an extracellular supply of thymidine or thymine. A second approach to the problem was to compare the effect on dTMP synthetase activity of infection by phages Øe and SP8. The latter phage was known to induce a dTTPase which would undoubtedly prevent the incorporation of thymine into the phage DNA (Kahan et.al., 1964).

Enzyme changes occurring after phage Øe infection

The original observation of Roscoe and Tucker (1966) that dTMP synthetase disappeared during the initial minutes of phage infection was repeated (Fig. 3.). A culture of B. subtilis 3610 in supplemented DMM was infected in early log phase with phage Øe at a multiplicity of mixing of 5 p.f.u. per viable bacterium, and subsequently chilled and harvested at various times after infection (Methods). Thymidylate synthetase and the phage-induced dUMP hydroxymethylase were assayed in crude cell-free extracts prepared by sonication; the latter enzyme was a useful indicator of the normality of the infection in any one experiment.

The decrease in dTMP synthetase activity was detectable five minutes after addition of phage Øe, and

Figure 3

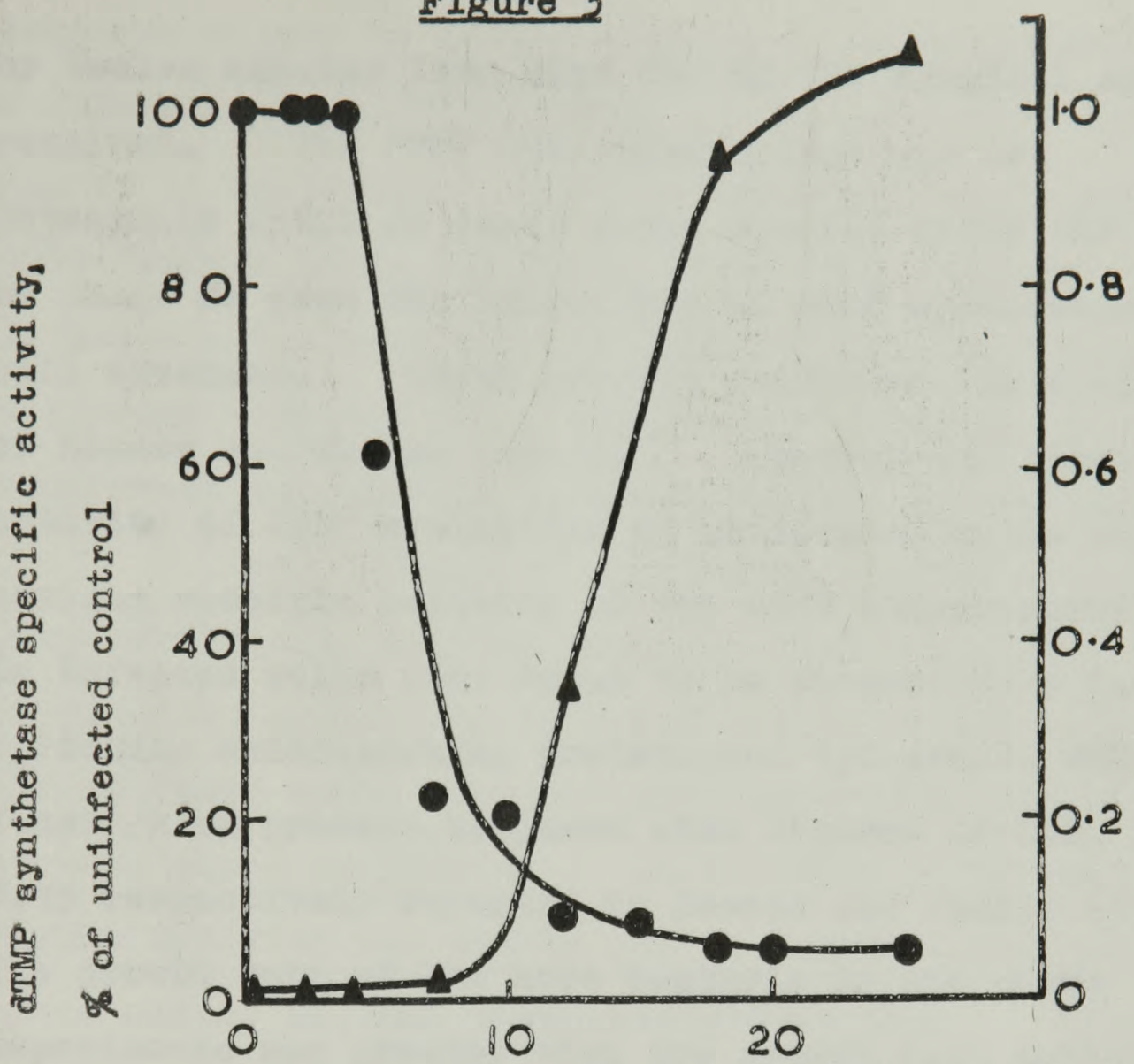


Fig. 3. Enzyme changes occurring after phage Øe-infection of B. subtilis 3610

Extracts were prepared from infected cultures at various times after the addition of phage Øe and assayed for

●——● dTMP synthetase , and ▲——▲ dUMP hydroxymethylase.

(The activity of dTMP synthetase in infected extracts was expressed as a percentage of the uninfected control because the latter varied slightly in different expts) .

by twelve minutes less than 10% of the original activity remained. The dUMP hydroxymethylase was not detectable until at least seven minutes after the addition of phage Øe when the inhibition of dTMP synthetase was well advanced. These results confirmed the findings of Roscoe and Tucker (1966). However the specific activity of dTMP synthetase in uninfected cells and the maximum specific activity of the dUMP hydroxymethylase in infected cells were found to be respectively 0.04 μmoles H_4PteGlu oxidised/h/mg protein and 1.0 μmoles dHUMP formed/h/mg protein, compared with figures of 0.02 and 0.15 respectively reported by Roscoe and Tucker (1966). The growth rate of the host bacteria in the above experiments was greater than the growth rate achieved using the conditions of Roscoe and Tucker (1966), due to the addition of ferric citrate to the medium. The change in growth rate was believed to be responsible for the increase in the specific activity of dTMP synthetase and at least partly responsible for the higher specific activity of the dUMP hydroxymethylase.

Effect of inhibiting protein synthesis on the disappearance of dTMP synthetase

Protein synthesis by the infected culture was necessary in order to observe the disappearance of dTMP synthetase. An inhibition of protein synthesis was

achieved either by adding 50 μg/ml of chloramphenicol to cultures of B. subtilis 3610 at the same time as phage Øe, or by infecting a tyrosine auxotroph of B. subtilis 3610, strain 3/13, which was starved of tyrosine. Infected cells were then chilled at various times, and cell-free extracts were prepared and assayed for dTMP synthetase and the dUMP hydroxymethylase in the usual way.

Both methods of inhibiting protein synthesis gave essentially similar results (Figs. 4 and 5). Thymidylate synthetase in uninfected cultures was stable for at least sixty minutes under conditions inhibiting its continued synthesis. In infected cultures the enzyme activity was stable for at least twenty minutes after adding Øe, and then slowly decreased. This response was quite different from the rapid decrease in dTMP synthetase activity obtained under normal conditions of infection. Moreover even the slow decrease in activity was prevented if both methods of inhibiting protein synthesis were combined (Fig. 5).

These results suggested that neither method of inhibiting protein synthesis completely prevented the synthesis of an inhibitor of dTMP synthetase, but that the methods were effective in combination. This was confirmed by a further experiment in which phage infection was allowed to proceed normally for five minutes and chloramphenicol then added (Fig. 4). The rate of loss of dTMP synthetase was considerably slowed as would

Figure 4

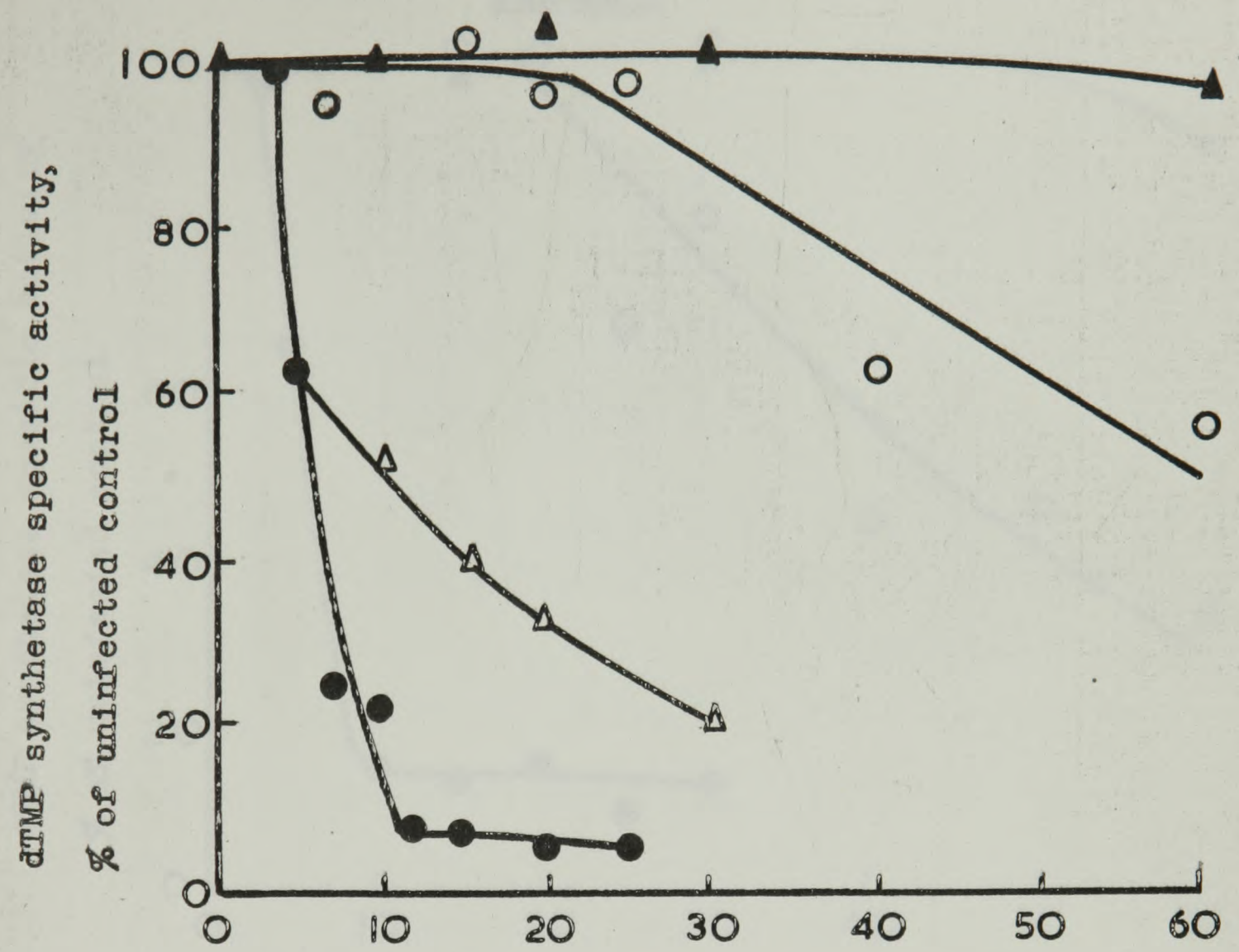


Fig. 4. Effect of chloramphenicol on the loss of dTMP synthetase activity

dTMP synthetase was measured in extracts prepared from cultures of B. subtilis 3610 at various times after the following additions.

▲—▲ 50 µg/ml of chloramphenicol added at t=0, no Øe

o—o 50 µg/ml of chloramphenicol and phage Øe added at t=0

△—△ Phage Øe added at t=0, 50 µg/ml of chloramphenicol added at t=5

●—● Phage Øe added at t=0, no chloramphenicol.

Figure 5

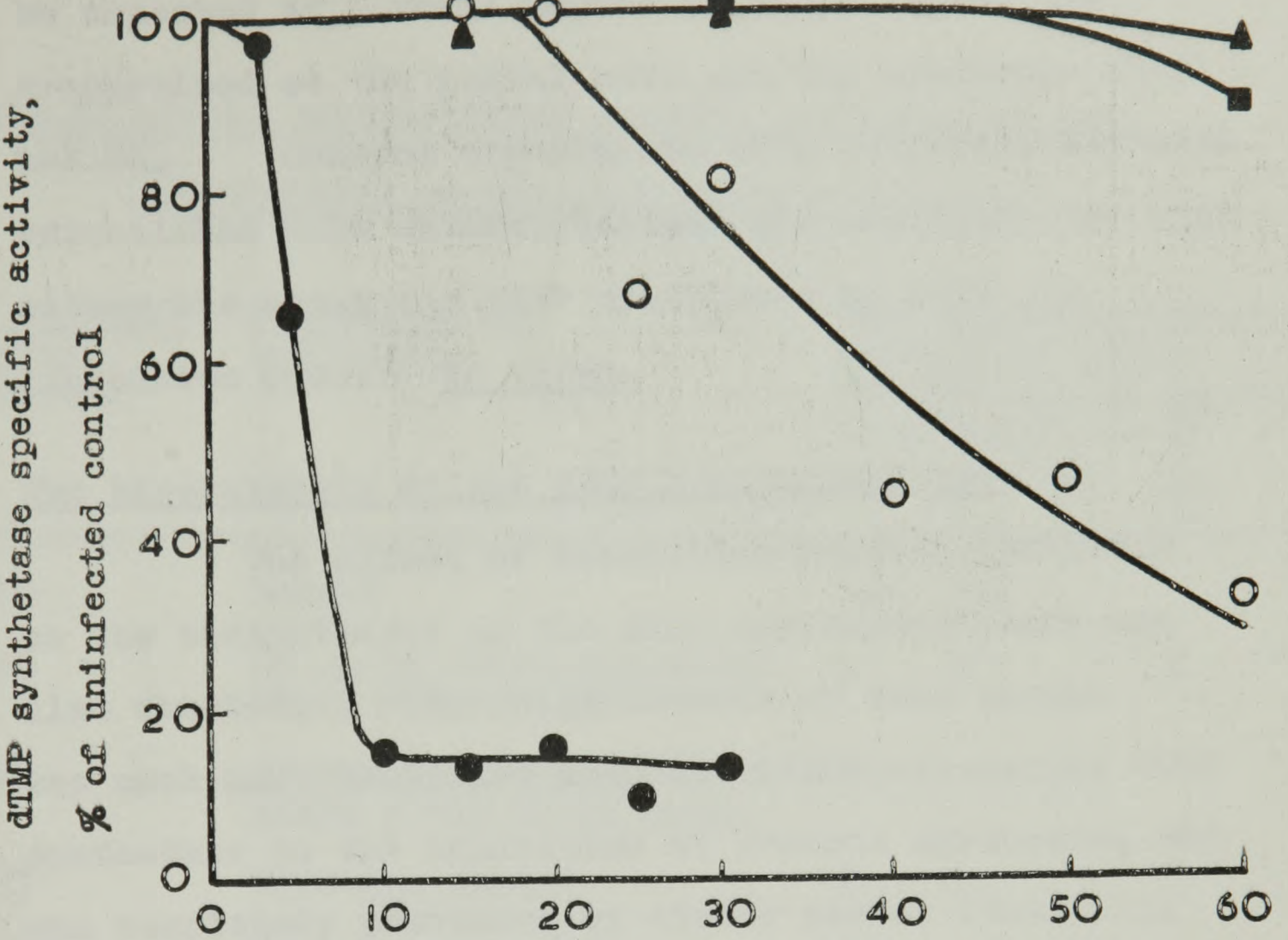


Fig. 5. Enzyme changes occurring after phage infection of B. subtilis 3.13 under various conditions

dTMP synthetase was measured in extracts of cultures which had been grown to $3 \cdot 10^8$ viable cells per ml in the presence of tyrosine, centrifuged, washed with DMM, resuspended in warm supplemented DMM minus tyrosine, equilibrated for 10 min at 37° and finally chilled at various times after the following additions.

▲—▲ Nothing added

■—■ 50 µg/ml of chloramphenicol and phage Øe added at t=0

○—○ Phage Øe added at t=0

●—● Phage Øe and tyrosine added at t=0.

be expected if a small amount of an inhibitor was synthesised at the normal rate and the synthesis then halted. Control experiments with cell-free extracts established that chloramphenicol did not interfere with either the assay for dTMP synthetase or with the inhibition process in vitro.

The biosynthesis of the dUMP hydroxymethylase

The effect of inhibiting protein synthesis on the biosynthesis of the dUMP hydroxymethylase was also checked. The biosynthesis of this enzyme was much more sensitive than the disappearance of dTMP synthetase to the inhibition of protein synthesis, and was completely prevented by either method (Table 2). Even when phage infection was allowed to proceed normally for five minutes no dUMP hydroxymethylase was detected, although considerable inhibition of dTMP synthetase occurred in this time.

Effect of inhibiting protein synthesis on the incorporation of ^{35}S into material insoluble in trichloracetic acid (TCA)

In an additional control experiment, which was designed to check the rate of protein synthesis under various conditions, the rate of incorporation of $(^{35}SO_4)^{=}$ into material insoluble in TCA was investigated. The results are shown in Fig. 6. Cultures of B. subtilis 3/13 starved of tyrosine incorporated $(^{35}SO_4)^{=}$ at only 3%

Table 2. Effect of inhibiting protein synthesis on the biosynthesis of the dUMP hydroxymethylase

B. subtilis strain infected	Conditions of infection	dUMP hydroxymethylase activities after various min of exposure to phage Øe at 37°			
		20	25	30	60
3610	Normal	+++	+++		
3610	50 µg/ml chloramphenicol added with phage	0	0		0
3610	50 µg/ml chloramphenicol added 5 min after phage	0		0	
3/13	Tyrosine absent	0	0		0
3/13	Tyrosine present	++	+++	+++	

Some of the extracts from the phage Øe-infected cultures prepared in the experiments described in Figs. 4 and 5 were assayed for dUMP hydroxymethylase activities as shown.

Figure 6

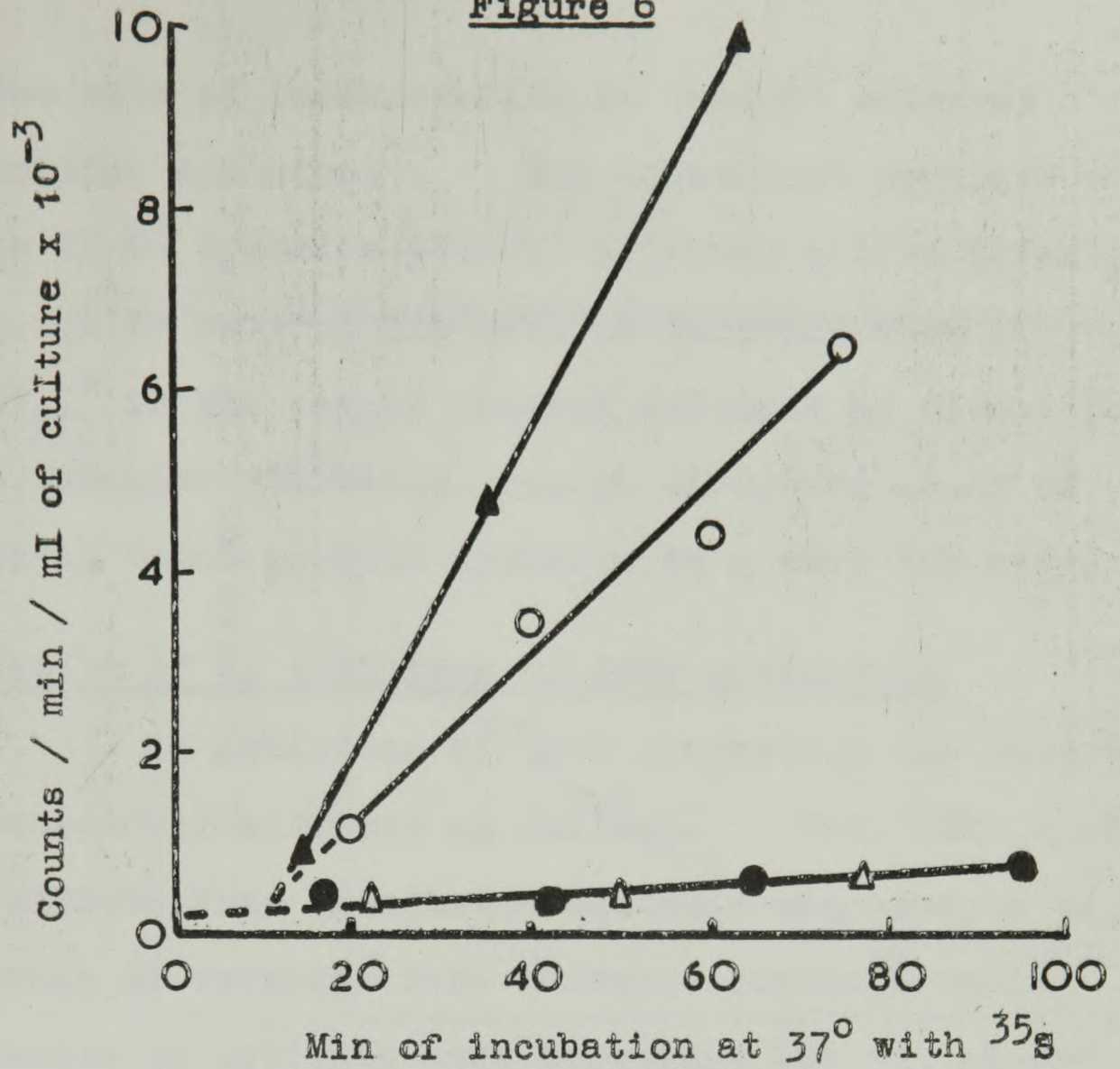


Fig. 6. Incorporation of $(^{35}SO_4)^{=}$ into material insoluble in TCA

A culture of B. subtilis 3.13 at $3 \cdot 10^8$ cells / ml was washed with, and resuspended in, the same volume of warm supplemented sulphate-free DMM minus tyrosine. 1.25×10^{-4} M sodium sulphate and approximately 0.4 µC /ml of carrier-free $(^{35}SO_4)^{=}$ were added and the culture was distributed in 9 ml batches to 5 T-tubes to which the following additions were made.

●——● None, Δ——Δ phage Øe added at t = 10,

▲——▲ tyrosine added at t = 10,

o——o tyrosine and phage Øe added at t = 10.

At intervals 1 ml samples from each T-tube were added to 1 ml of ice-cold TCA (10%) . After standing for at least 30 min, samples of the precipitate were collected on a Millipore filter type HA, dried with an infra-red lamp and counted using an end-window counter (Methods).

of the rate of incorporation in control cultures containing tyrosine. The additional presence of phage Øe in tyrosine-starved cultures had no effect, although it reduced the rate of incorporation of $(^{35}SO_4)^=$ in the normal control cultures by almost 50%. Thus tyrosine starvation was an effective means of reducing total protein sythesis to a very low rate.

Detection of an inhibitor of dTMP synthetase

An inhibitor of dTMP synthetase was detected in infected-cell extracts as follows. When dTMP synthetase in extracts from uninfected bacteria was assayed in the presence of extracts from infected bacteria small decreases in activity were sometimes but not always observed. A more reproducible inhibition was obtained by incubating the mixed extracts at 37° before assaying for dTMP synthetase. This inhibition increased progressively with increasing duration of the incubation at 37° (Fig. 7), but there was no perceptible further loss of enzyme in partially inhibited mixed extracts at the temperature and dilution of the dTMP synthetase assay. Control experiments showed that dTMP synthetase was stable when uninfected-cell extracts were incubated alone at 37°.

This observation was used to develop the following assay for the inhibitor. A sample of dTMP synthetase from uninfected B. subtilis was incubated with

Figure 7

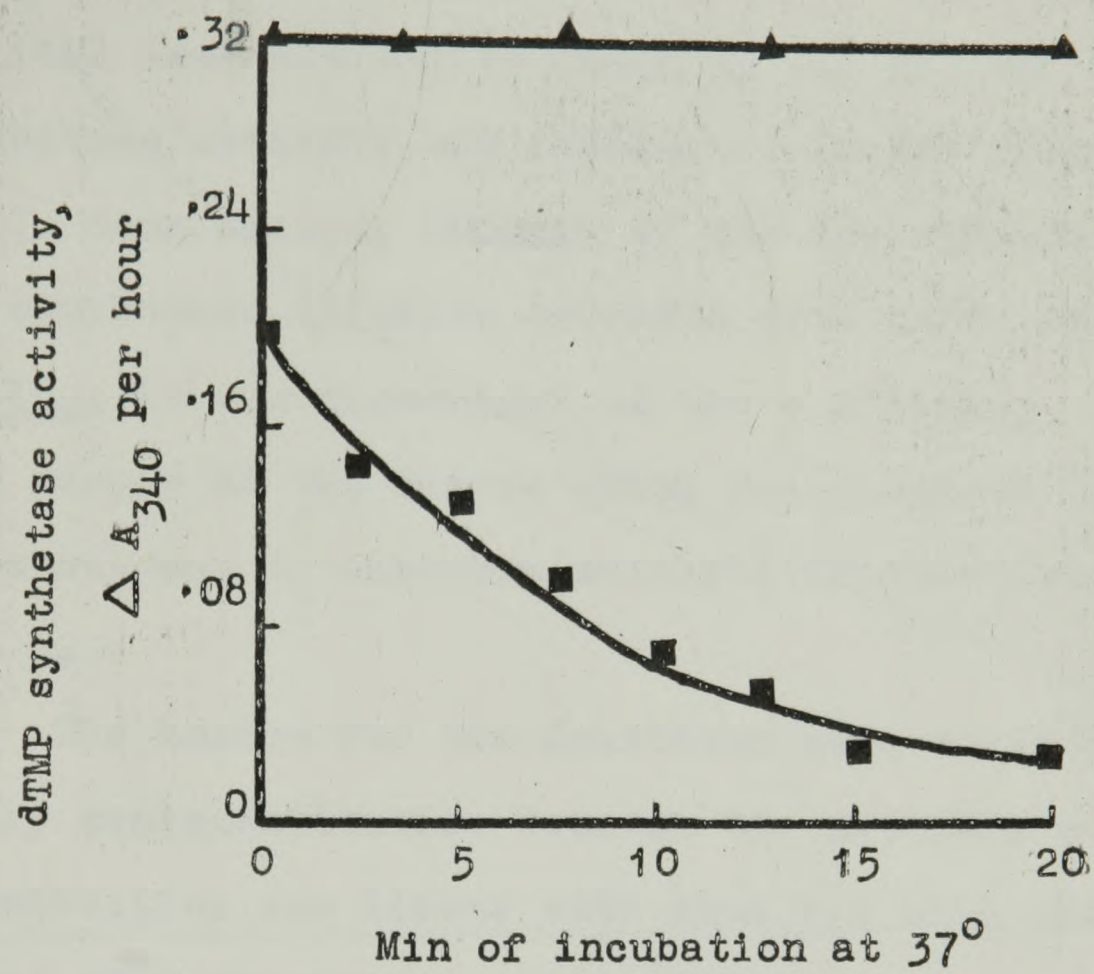


Fig. 7. Inhibition of dTMP synthetase in vitro

■—■ A 0.2 ml sample of the cell-free extract from uninfected B. subtilis was mixed with an equal volume of an extract from B. subtilis harvested 25 min after the addition of phage Øe. The mixture was incubated at 37° for various lengths of time before assaying 0.1 ml for dTMP synthetase activity.

▲—▲ A 0.4 ml sample of the extract from uninfected cells was similarly incubated alone at 37° before assaying 0.1 ml for dTMP synthetase activity.

(The dTMP synthetase activity of the individual samples used was as follows;
ΔA_{340} /h for 0.1 ml of uninfected extract was 0.317
ΔA_{340}/h for 0.1 ml of infected extract was 0.035).

a sample of infected-cell extract at 37° in a constant volume (usually 0.5 ml) for five or ten minutes. The incubation was terminated by diluting and chilling the mixture with ice-cold buffer (usually 0.5 ml) and the residual dTMP sythetase activity was determined in the usual way. In some assays, because of the low specific activity of dTMP synthetase in crude extracts from uninfected B. subtilis, it was convenient to use a slightly purified sample of the enzyme which was prepared from the crude extract by ammonuim sulphate fractionation (page 59).

The assays for the inhibitor were considered to be only semiquantitative because the progress curve of the inhibition was linear with time and with the amounts of dTMP synthetase and inhibitor over a rather limited range of conditions (described in Chapter II) which were not necessarily achieved in the following experiments. Therefore no attempt was made at this stage to calculate a 'specific activity' for the inhibitor, but where relevant, the protein concentrations of inhibiting samples were included in the results. The assay was useful for a preliminary study of the inhibitor.

Properties of the inhibitor of dTMP synthetase

The properties of the inhibitor demonstrated in the following experiments all indicated that it could be a protein. The inhibitor was not dialysable (Fig. 8)

Figure 8

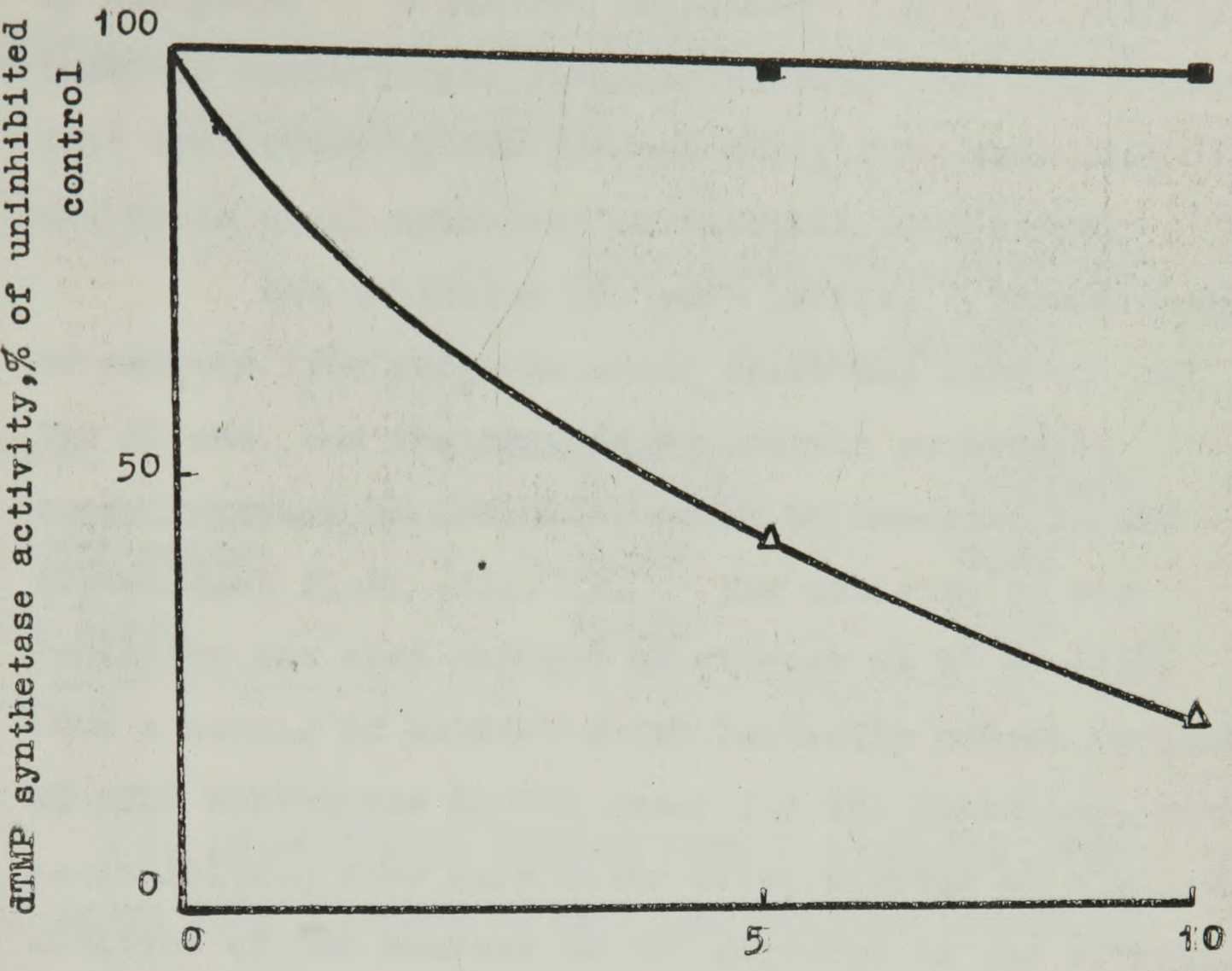


Fig. 8. Properties of the inhibitor of dTMP synthetase

Extracts from phage-infected cells were treated as described below and then assayed for the presence of the inhibitor by incubating a sample with an equal volume of dTMP synthetase from uninfected cells.

■—■ The infected extract was boiled (30 sec at 100°) and the supernatant fluid assayed for the inhibitor.

Δ—Δ The infected extract was dialysed over-night against two changes of buffer A and then assayed for the inhibitor.

and dTMP synthetase in infected-cell extracts was not restored by dialysis. A control experiment (Table 3) established that the conditions of dialysis reversed the inhibition of dTMP synthetase by its product dTMP, thus excluding this and other small molecules as possible inhibitors.

The inhibitor was very labile. When a sample of extract from phage-infected cells was held at 100° for 30 sec. and the coagulated protein removed by centrifugation, no inhibitor could be detected in the supernatant fluid (Fig. 8). The activity of the inhibitor was also reduced by storage at 4° or -15°. Thus a sample of extract which initially caused 86% inhibition of dTMP synthetase in the assay for the inhibitor, caused no inhibition five days later after storage at 4°. The addition of 10% sucrose or 10% glycerol to the extract considerably improved the stability of the inhibitor towards storage at both temperatures and in later experiments 10% glycerol was routinely used for this purpose. Thus the sample of inhibitor quoted above lost no activity when stored at 4° for 5 days in the presence of 10% glycerol.

The inhibitor could be precipitated by enzyme-grade saturated ammonium sulphate solution (Table 4), and the appropriate ammonium sulphate fraction was conveniently used to investigate the effect of various hydrolytic enzymes on the inhibitor. The inhibitor was destroyed by

Table 3. Inhibition of dTMP synthetase by dTMP and its reversal by dialysis

Sample	dTMP synthetase activities ΔA_{340} per hour	
	Before dialysis	After dialysis
No dTMP added	0.128	0.09
dTMP added	0.034	0.09

2 ml of cell-free extract from B. subtilis 3610 was mixed with 8 µmoles of dTMP and dialysed overnight against two changes of buffer A. A similar sample containing no added dTMP was also dialysed. Equal volumes (0.1 ml) of both samples were assayed for dTMP synthetase activity before and after dialysis.

Table 4. Precipitation of inhibitor by ammonium sulphate

Sample tested for inhibitor	Residual dTMP synthetase activity after 10 min at 37°	% inhibition	Vol of fraction	Protein concn.
	ΔA_{340} / h		ml	mg / ml
Control dTMP synthetase	0.48	0		
Unfractionated extract	0.20	58	8.5	9
0-40% saturation fraction	0.04	92	4.0	4
40-60% saturation fraction	0.50	0		
Dialysed supernatant from above	0.46	0		

Cell-free extracts prepared from a phage-infected culture of B. subtilis 3610 25 min after addition of phage Øe, were fractionated with 'enzyme-grade' saturated ammonium sulphate solution. The precipitates were resuspended in buffer A and dialysed before incubating 0.4 ml samples with dTMP synthetase to test for inhibitory activity.

fifteen minutes' incubation at 37° and pH 7.5 with 20 μg/ml of trypsin (Table 5) but was not significantly affected by ribonuclease (20 μg/ml), or pancreatic deoxyribonuclease I (20 μg/ml in the presence of 5mM Mg Cl_2) under these conditions.

Appearance of the inhibitor after phage infection

To show that the amounts of inhibitor present in infected-cell extracts increased during the course of the infection, extracts from bacteria which had been chilled at different times after infection were assayed for inhibitor. In addition to experiments using *B. subtilis* 3610 these experiments were repeated using as host strain *B. subtilis* 168T⁻. The latter strain was deficient in dTMP synthetase activity (Table 7) and the assays for the inhibitor were therefore not complicated by the presence of small, variable amounts of dTMP synthetase activity derived from infected cells.

During infection of *B. subtilis* 3610 the inhibitor was detectable five minutes after the addition of phage Øe and was present in increased amounts at fifteen and twenty-five minutes (Table 6). During infection of *B. subtilis* 168T⁻ the amount of inhibitor increased rapidly between five to ten minutes after adding phage Øe and thereafter more slowly (Fig. 9). In both cases the inhibitor behaved like an early phage-induced function. The appearance of inhibitor in extracts from phage-

Table 5. Destruction of inhibitor of dTMP synthetase by trypsin

Incubation at 37° in buffer A	Time (min)	Residual dTMP synthetase activity ΔA_{340} / h
Effect of trypsin on dTMP synthetase		
dTMP synthetase control	10	0.73
dTMP synthetase and 10 µg trypsin	15	0.07
dTMP synthetase, 10 µg trypsin and 22 µg trypsin inhibitor	15	0.71
Effect of trypsin on phage Øe inhibitor		
Phage Øe inhibitor alone	15	
then with dTMP synthetase	10	0.32
Phage Øe inhibitor and 10 µg trypsin,	15	
then with dTMP synthetase and 22 µg trypsin inhibitor	10	0.72
Phage Øe inhibitor, 10 µg trypsin and 22 µg trypsin inhibitor,	15	
then with dTMP synthetase	10	0.38

A sample of phage Øe inhibitor was incubated alone or with trypsin ± trypsin inhibitor in a total volume of 0.4 ml for 15 min; 0.1 ml of a sample of dTMP synthetase was added for a second incubation to assay for the inhibitor. Appropriate ammonium sulphate fractions containing inhibitor or dTMP synthetase were used in these experiments. The control experiment on dTMP synthetase was used to establish the necessary balance of trypsin and trypsin inhibitor.

Table 6. Appearance of inhibitor in phage-infected extracts

of B. subtilis 3610

Min of exposure to phage ∅e at 37°	Test for inhibitor. Residual dTMP synthetase activity ΔA_{340} / h		% inhibition	Protein concn. of infected samples (mg/ml)
	Unincubated	Incubated		
Expt. 1.				
Control enzyme	0.76	0.74	0	4.6
5	0.95[x]	0.76	20	4.6
15	0.73	0.34	53	5.2
Expt. 2.				
Control enzyme	0.65	0.65	0	
25	0.60	0.24	60	4.4

Equal volumes (0.3 ml) of extracts from infected cultures chilled at different times after addition of phage ∅e, and control enzyme from uninfected B. subtilis were mixed and the residual dTMP synthetase assayed both with and without 10 min incubation at 37°. The percentage inhibition was calculated from the difference in activities of mixed extracts before and after incubation.

x The high activity was due to a significant amount of residual dTMP synthetase in the infected extract.

Figure 9

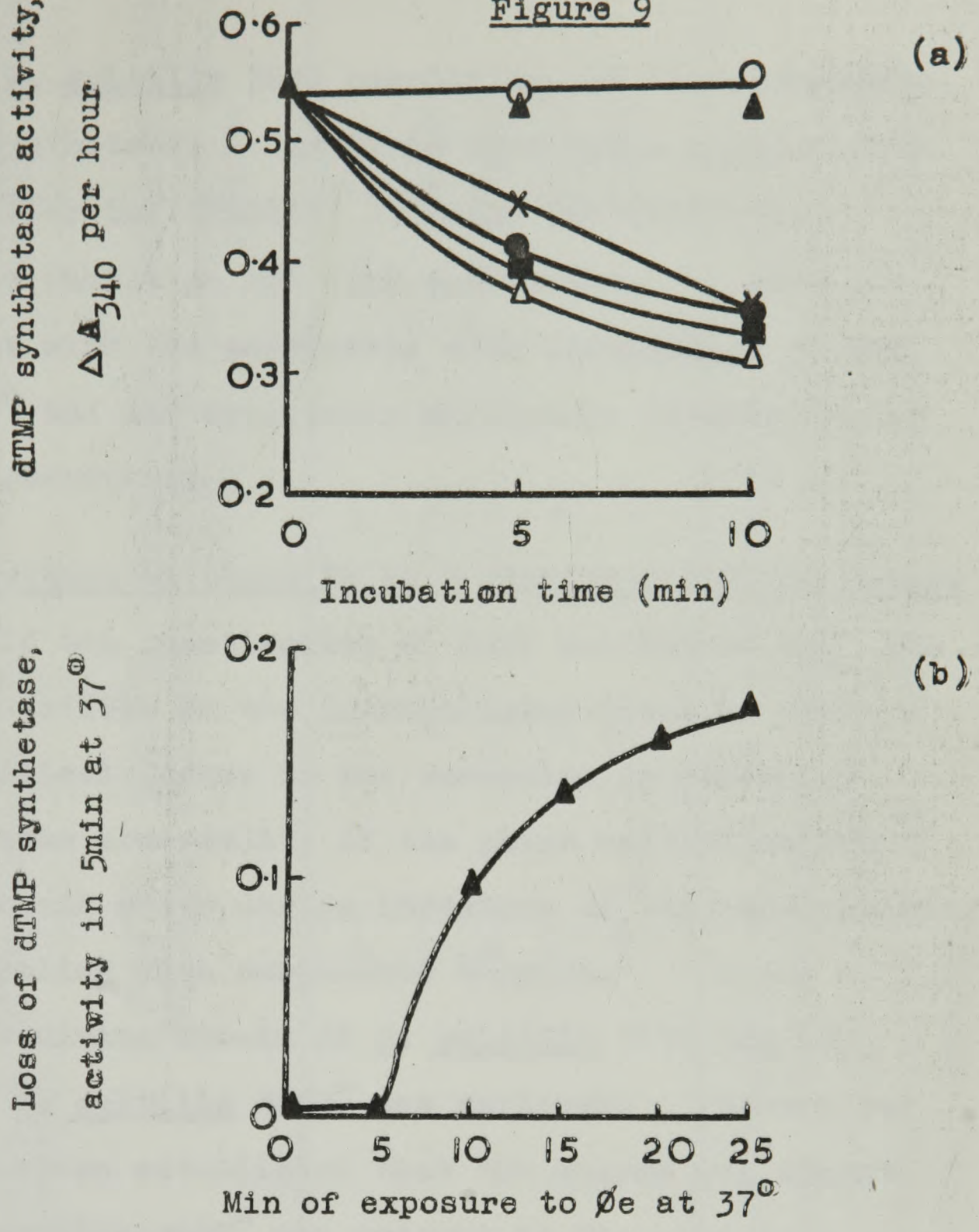


Fig. 9a. Appearance of the inhibitor in phage-infected B. subtilis 168T-

0.3ml of extracts from infected cultures chilled at various times after the addition of phage Øe were incubated at 37° with a constant volume of a dialysed ammonium sulphate fraction containing dTMP synthetase. The incubations were terminated after 5 or 10 min by dilution with chilled buffer A, and the residual dTMP synthetase was assayed.

▲——▲ Uninfected control; o——o , ×——× , ●——● , ■——■ , and Δ——Δ, were respectively extracts from cells chilled 5,10, 15,20,and 25 min after the addition of Øe.

Fig. 9b. shows the data of Fig. 9a replotted to illustrate the appearance of the inhibitor.

infected B. subtilis 3610 paralleled the disappearance of dTMP synthetase. Extracts from cells chilled five minutes after the addition of phage contained both detectable inhibitor and dTMP synthetase. This was consistent with the measurably slow interaction of the two at 37° and the apparently negligible interaction at lower temperatures.

The replication of phage Øe in a thymine-requiring mutant

If the inactivation of dTMP synthetase and its resultant effect on the intracellular level of dTTP were a critical factor in the succesful infection of phage Øe some abnormality in the phage multiplication process should occur during infection of a thymine-requiring mutant supplied with exogeneous thymine. Since a thymine-requiring strain of B. subtilis 3610 was not available B. subtilis 168T⁻ was employed. Assays for dTMP synthetase established that the enzyme was absent from B. subtilis 168T⁻ and present in the wild type B. subtilis 168 although at a lower specific activity than in B. subtilis 3610 (Table 7).

The normality of the infection of B. subtilis 168T⁻ by phage Øe in the presence of exogeneous thymidine was demonstrated by a number of criteria. A stimulated rate of DNA synthesis subsequent to infection was terminated by cellular lysis, and accompanied by a large increase in p.fu. at about 50 min. after the addition

Table 7. Thymidylate synthetase activity in different strains of B. subtilis grown in the presence and absence of thymidine

B. subtilis strain	Addition to 40 ml of overnight culture in nutrient broth plus M/200 glucose	Additions to growth medium of 100ml of supplemented DMM			dTMP synthetase specific activity
	$2 \cdot 10^{-4}$M Thymidine	$1 \cdot 10^{-4}$M Thymidine	$5 \cdot 10^{-4}$M Tryptophan	0.05% acid-hydrolysed casein	µmoles of H_4PteGlu oxidised / h / mg protein
168 T⁻	+	+	+	+	Nil
168	+	+	+	+	0.025
168	-	-	+	+	0.020
3610	+	+	-	+	0.039
3610	-	-	-	+	0.036

The bacteria from five overnight cultures of the three different strains of B. subtilis were centrifuged, washed with DMM and resuspended in 4 ml of DMM. A 1 ml inoculum of each suspension was added to 100 ml of supplemented DMM and the cultures were grown to an EEL colorimeter reading of 2.0 . They were then chilled, harvested, and assayed for dTMP synthetase activity.

of phage Øe (Fig. 10). In addition, an experiment described earlier in this chapter showed that the inhibitor of dTMP synthetase was induced during the multiplication of phage Øe in B. subtilis 168T⁻ (Fig. 9).

Since phage multiplication was normal it was apparent that either thymine nucleotides were efficiently excluded from phage Øe DNA by a mechanism which was not bypassed by an obligatory external supply of thymidine, or that the incorporation of thymine into phage DNA did not affect the viability of the phage.

The incorporation of thymidine into phage DNA

The incorporation of thymidine into the DNA of phage Øe during multiplication in B. subtilis 168T⁻ was investigated as follows. B. subtilis 168T⁻ was infected with phage Øe and the progeny phage were purified by differential centrifugation (Methods). The DNA from the phage was isolated, purified and hydrolysed enzymatically to mononucleotides (Methods). The latter were separated by paper chromatography.

The resultant chromatogram is shown in Fig. 11. Only four ultraviolet-absorbing spots were visible. The pyrimidines were identified as dCMP and dHUMP by their position on the chromatogram and by their ultraviolet optical densities in 0.1NHCl (Fig. 11). A marker of dTMP was co-chromatographed with one sample of the DNA hydrolysate to indicate the position of thymidylic acid.

Figure 10

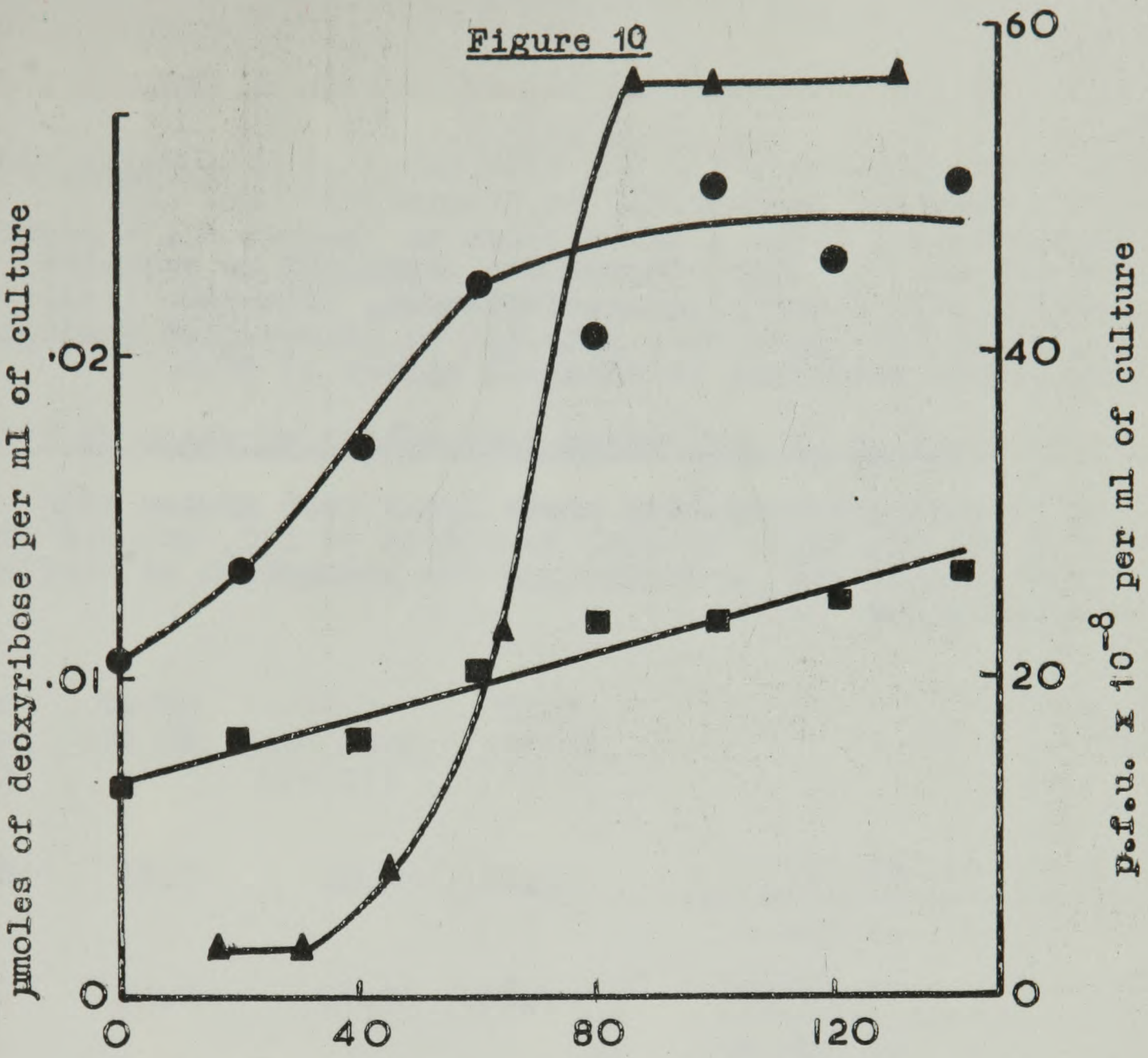


Fig. 10. The replication of phage Øe in B. subtilis 168 T⁻

▲—▲ A 10 ml culture of *B. subtilis* 168T⁻ was infected with phage Øe. The culture was diluted 1x10⁶ into an identical growth medium and sampled at intervals for p.f.u. A correction was made for unadsorbed phage.

●—● A 200 ml culture of *B. subtilis* 168T⁻ was infected with phage Øe. Duplicate 7.5 ml samples were removed at intervals, added to 0.75 ml of ice-cold TCA (50%) , and assayed for DNA (Methods).

■—■ A parallel uninfected culture was similarly assayed for DNA.

Figure 11. The chromatographic separation of the nucleotides of phage Øe DNA

DNA from phage Øe which had been grown in B. subtilis 168T⁻ was hydrolysed enzymically to mononucleotides. The hydrolysate (0.5ml) was applied to Whatman No. 1 paper at the origin. The chromatogram was developed by downward elution with 2-methylpropan-2-ol-conc. HCl-water (11:1:3 by vol.) and the spots were detected by ultraviolet photography. One sample contained an internal marker of dTMP.

Identification of pyrimidine nucleotides of phage Øe DNA

The ultraviolet-absorbing paper areas were eluted into 0.1 N HCl and their optical densities at 250, 260 and 280 mμ were measured and corrected for the absorption of similarly-treated paper blanks.

	dCMP (Øe DNA)	dCMP (published figures)	dHUMP (Øe DNA)	dHUMP (published figures*)
Absorption at 250 mμ	0.44	0.43	0.73	0.71
" at 260 mμ				
" at 280 mμ	2.12	2.12	0.56	0.56
" at 260 mμ				

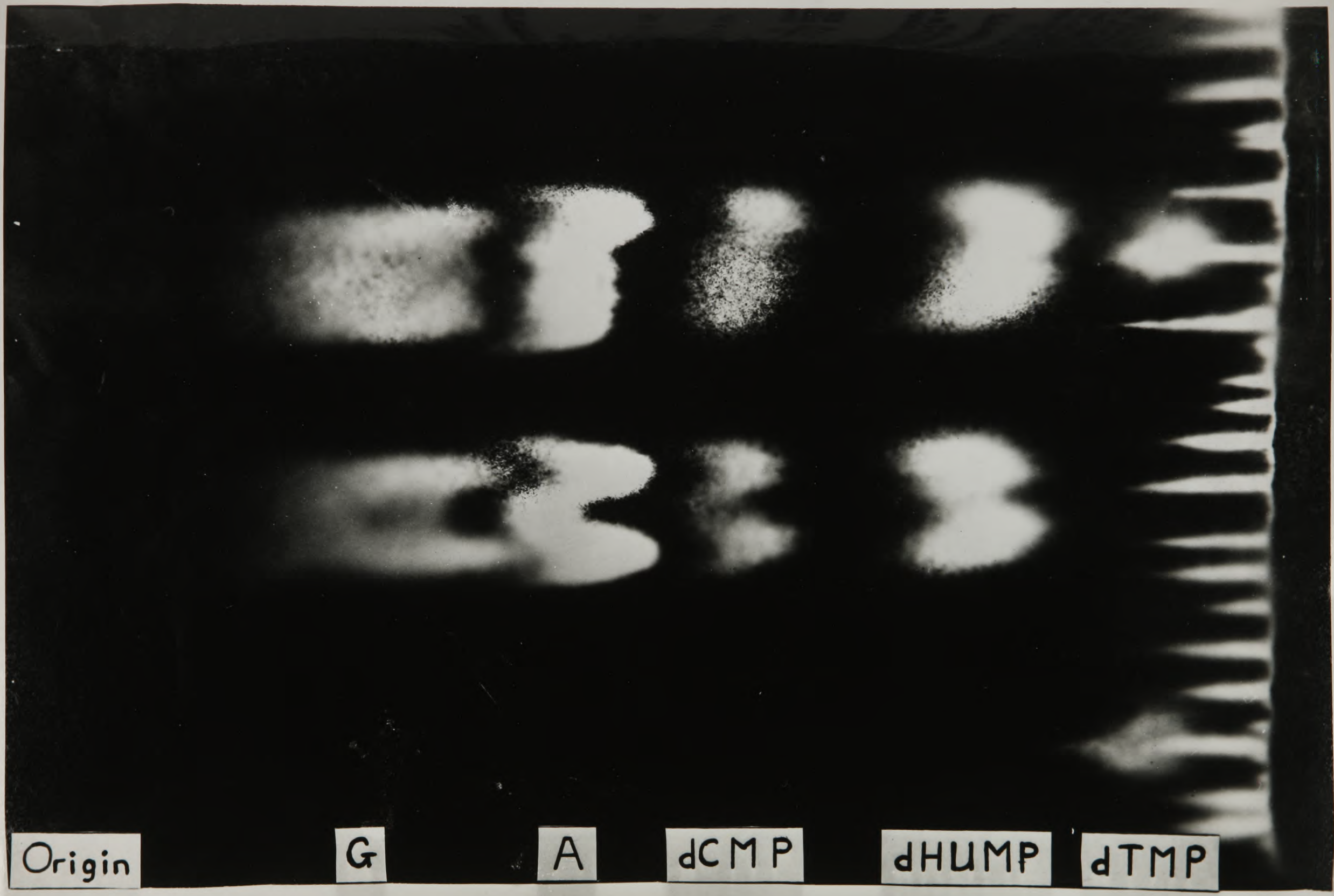
*Takahashi and Marmur (1963)

Relative quantities of pyrimidine nucleotides in phage Øe DNA

The amount of dHUMP was calculated from the optical density at 264 mμ using the value of 10.2 for the millimolar extinction coefficient (Kuno and Lehman, 1962).

Expt.	μmoles dHUMP / μmoles dCMP
1	1.24
2	1.18
3	1.20
Roscoe (1963)	1.40

A= adenine derivative
G= guanine derivative


Origin
G
A
dCMP
dHUMP
dTMP

There was no evidence from the chromatogram that the phage DNA contained dTMP. However, the amount of dHUMP detected was somewhat low compared with the amount found by Roscoe (1965) using the same method. Although the lability of dHUMP might account for the discrepancy it was also possible that it was due to a small incorporation of dTMP. The latter possibility was examined in a preliminary experiment in which the phage were grown in the presence of (2-^{14}C) thymidine and the incorporation of the label into the phage DNA nucleotides was determined. A figure for the maximum thymine content of phage DNA of 0.1% of the total bases was obtained, but further experiments were required to decide whether even this low incorporation was due to a possible contamination of phage DNA with bacterial DNA.

The infection of B. subtilis 168 by phage SP8

It was of interest to determine whether phage SP8, which also contains hydroxymethyluracil in its DNA (Kallen et.al., 1962), caused any decrease in dTMP synthetase activity after infection. Since phage SP8 was unable to multiply in B. subtilis 3610 (although it lysed this strain) the experiments were performed using B. subtilis 168. Under identical conditions and at the same multiplicity of infection phage Øe had a latent period of 55 - 60 min and phage SP8 had a latent period of

Figure 12

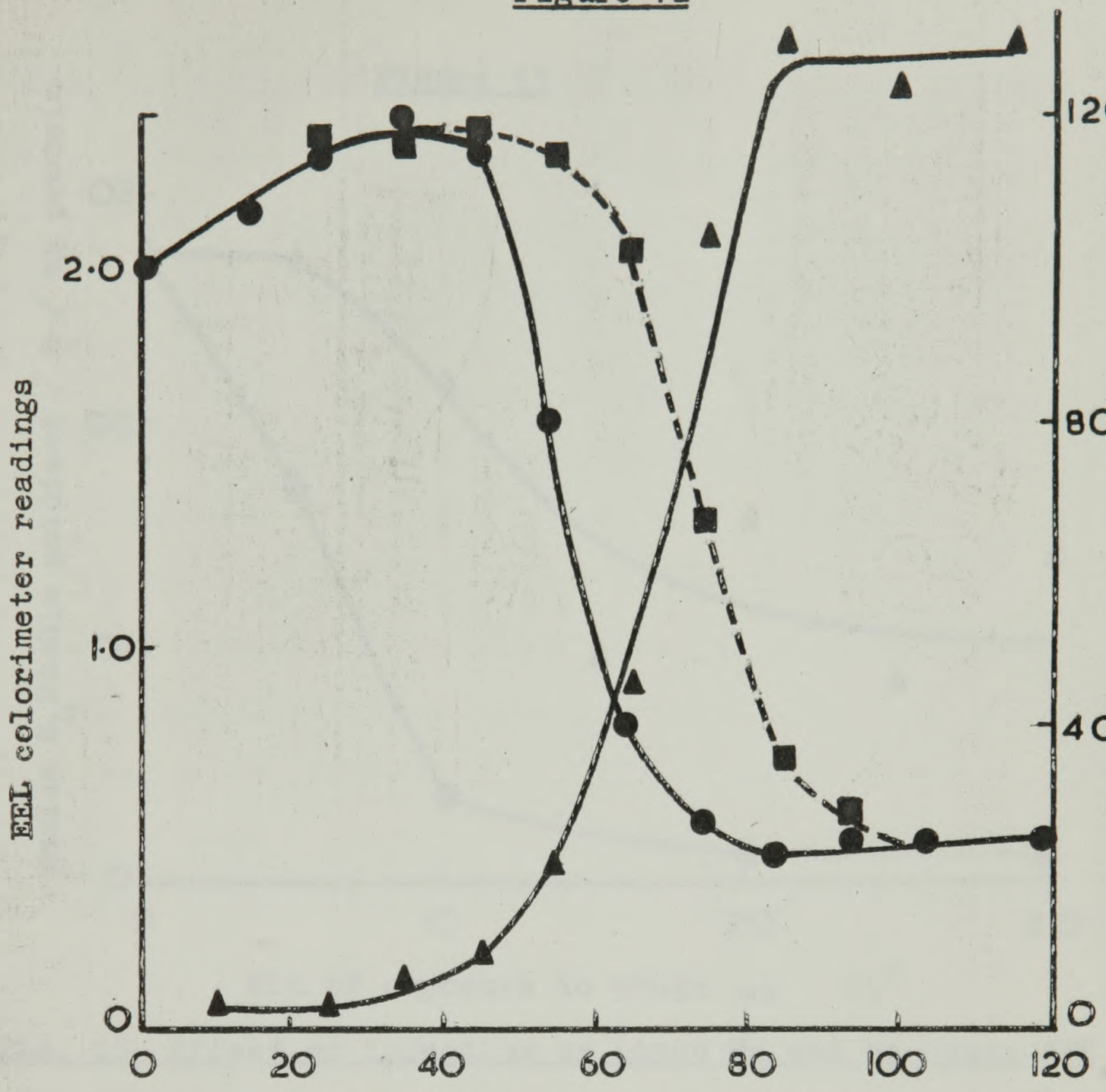


Fig. 12. The replication of phage SP8 in B. subtilis 168

Parallel 10 ml cultures of B. subtilis 168 were infected with either phage SP8 or phage Øe. The SP8-infected culture was diluted $1x10^6$ into an identical growth medium and sampled at intervals for p.f.u. A correction was made for unadsorbed phage.

●—● EEL colorimeter readings of SP8-infected culture

■—■ EEL colorimeter readings of Øe-infected culture

▲—▲ p.f.u.

Figure 13

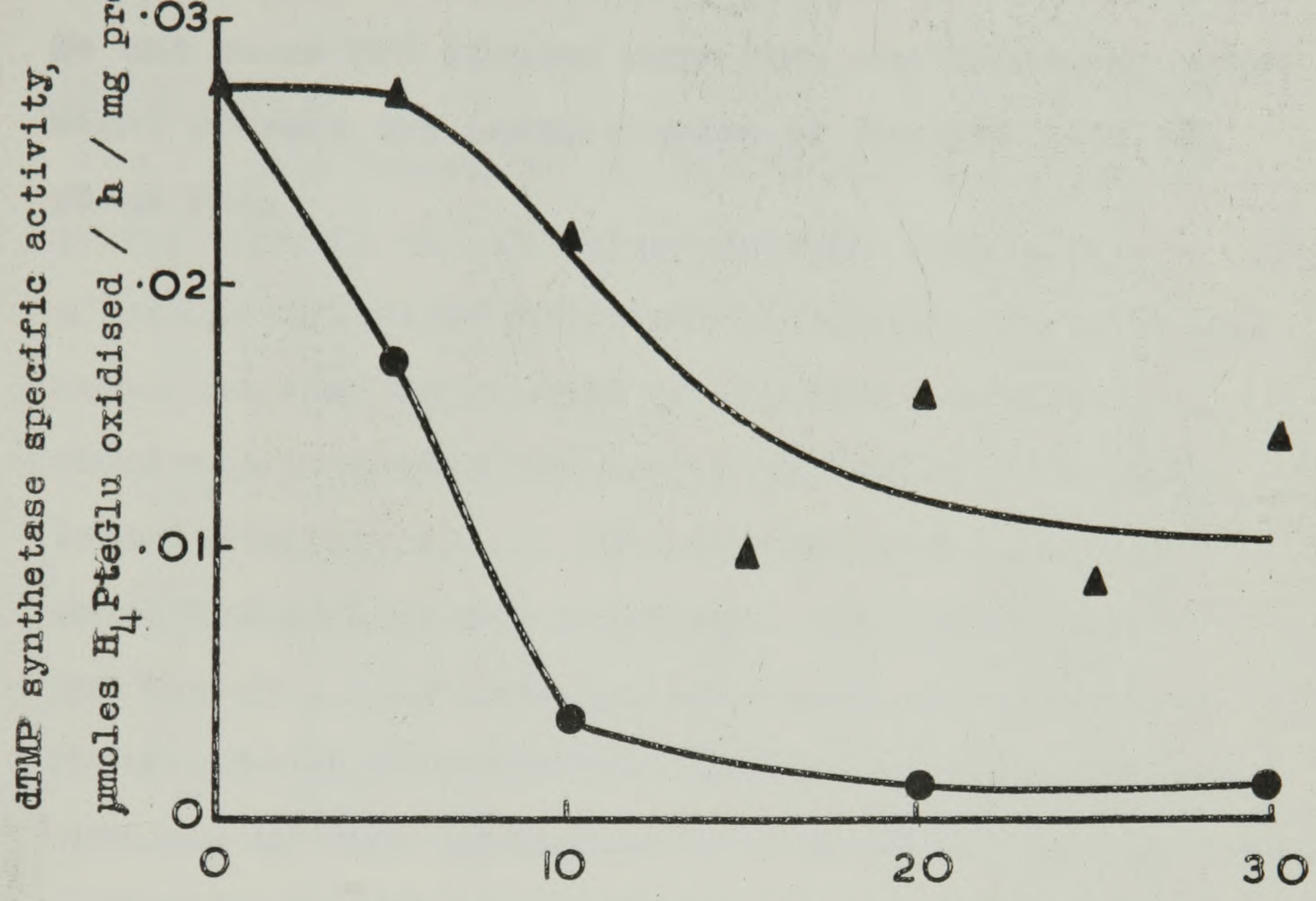


Fig. 13. Effect of infection by phage Øe and by phage SP8 on dTMP synthetase of B. subtilis 168

dTMP synthetase activity was measured in cell-free extracts which were prepared from cultures of B. subtilis 168 at various times after the addition of phage.

●——● Phage Øe ▲——▲ Phage SP8

of dTMP synthetase during infection of B. subtilis 168. Taken together these results suggested that both phage Øe and phage SP8 induced more than one mechanism which might prevent the incorporation of thymine into the phage DNA.

CHAPTER II The interaction between dTMP synthetase and the inhibitor

Introduction

The remainder of this thesis is devoted to an examination of the inhibitor of dTMP synthetase. Since a protein inhibitor might act by interfering with the assay for dTMP synthetase it was first necessary to eliminate this possibility. The kinetics of the inhibition process and the effects of varying the concentrations of the reactants, the temperature, and the pH of the reaction were then investigated in an attempt to determine whether the inhibitor acted directly on dTMP synthetase by a catalytic or stoichiometric mechanism. (The term stoichiometric is used in this context to mean the formation of a complex of the inhibitor and dTMP synthetase).

The isolation of purer samples of dTMP synthetase and its inhibitor.

In order to investigate the interaction of the inhibitor with dTMP synthetase in detail over a range of conditions it was decided to obtain both a purified sample of dTMP synthetase and a concentrated sample of the inhibitor free from the small amounts of residual dTMP synthetase. The purifications reduced the problem of

the precipitation that occurred in the cuvettes during assays for dTMP synthetase (Methods). An additional benefit from the purifications was that the concentrated samples thus obtained facilitated the design of a variety of experiments.

Thymidylate synthetase was purified 3-4 fold by treating extracts from uninfected cells with streptomycin sulphate to remove nucleic acids (Methods), followed by precipitation with ammonium sulphate (page 59) to select the fraction containing the enzyme (Table 8, line1). The inhibitor was purified from extracts of infected cells which were harvested 25 min after infection (Methods,.and page 66). It precipitated from the cell-free extracts at a much lower level of ammonium sulphate saturation than the low residual dTMP synthetase activity and could therefore be separated from the latter by this method (Table 9, line1). The ammonium sulphate precipitates of dTMP synthetase and inhibitor thus obtained were dissolved in a small volume of buffer D, (approx. 0.6 ml per litre of original culture), dialysed overnight and were then ready for use. It was usual to use the fractions immediately, although frozen samples of both inhibitor and dTMP synthetase retained at least 70% of their original activity, because of the stabilising effect of the glycerol in buffer D.

Table 8. Fractionation designed to isolate dTMP synthetase

Extract	$(NH_4)_2SO_4$ fraction % saturation	Specific activity of dTMP synthetase μmoles H_4PteGlu oxidised/h/mg protein	Specific activity of dUMP hydroxymethylase μmoles dUMP formed / h / mg protein	Presence of inhibitor
1. Normal uninfected extracts	0-60	0.013		
	60-90	0.116		
2. Equal amounts of 1 and 3 mixed before streptomycin sulphate treatment	0-60	0.008	0.6	
	60-90	0.065	0.2	
3. 20 min phage-infected extracts	0-60	0.001	1.0	Absent
	60-90	0.017	0.1	Absent

Extract from uninfected cells (protein concn. approx. 10 mg/ml) was fractionated with solid ammonium sulphate. The precipitates were resuspended in buffer D, dialysed and assayed for dTMP synthetase. Extract from infected cells which had been treated with streptomycin sulphate before centrifuging at 100 000 x g was similarly fractionated and assayed for dTMP synthetase, its inhibitor and the dUMP hydroxymethylase.

(Initial specific activity of dTMP synthetase: Uninfected-cell extracts= 0.045 μmoles H_4PteGlu oxidised /h/mg protein; Infected-cell extracts = 0.005 μmoles /h/mg).

Table 9. Fractionation designed to isolate the inhibitor of dTMP synthetase

Extract	$(NH_4)_2SO_4$ fraction	Specific activity of dTMP synthetase	Specific activity of dUMP hydroxymethylase	Presence of inhibitor
	% saturation	μmoles H_4PteGlu oxidised/h/mg protein	μmoles dUMP formed / h / mg protein	
1. 20 min phage-infected extracts	0-40	0.001	0.4	Present
	Supernatant	0.001	1.2	Absent
2. Equal amounts of 1 and 3 mixed without streptomycin sulphate treatment	0-40	0.001		Present
3. Normal uninfected extracts	0-40	0.002		Absent

Extract from infected cells (protein concn. approx. 10 mg/ml) was treated with 'enzyme-grade' saturated ammonium sulphate solution. The precipitate was dissolved in buffer D, dialysed and assayed for dTMP synthetase, its inhibitor and dUMP hydroxymethylase activities. Extract from uninfected cells was centrifuged at 100 000 x g without any treatment with streptomycin sulphate and the supernatant fluid similarly fractionated.

(Initial specific activity of dUMP hydroxymethylase in infected-cell extracts = 1.0 μmoles dUMP formed / h / mg protein).

Fractionation experiments

The above procedures for isolating dTMP synthetase and the inhibitor were utilised to show that the inhibitor did not act by masking or invalidating the assay for dTMP synthetase. Thus in the unfractionated extract from infected cells an apparent inhibition might be caused by an enzyme such as the dUMP hydroxymethylase removing one or both of the substrates of dTMP synthetase. This possibility was disproved by the following experiments which showed that there was no correlation in the fractionation behaviour of the inhibitor and the dUMP hydroxymethylase, and that dTMP synthetase activity in infected-cell extracts was not restored by a fractionation which separated it from the inhibitor.

Extracts from infected cells were processed by the method designed to isolate dTMP synthetase (Table 8, line 3). Although the fractionation both destroyed the inhibitor (presumably because of the use of solid ammonium sulphate) and separated dTMP synthetase from the ammonium sulphate fraction likely to contain undestroyed traces of inhibitor, there was no reactivation of dTMP synthetase. The small amount of residual enzyme was purified with the same percentage yield and increase in specific activity as the dTMP synthetase from uninfected cells.

The converse experiment of fractionating extracts from uninfected cells according to the method designed to isolate the inhibitor showed that the latter was not detectable prior to phage infection (Table 9, line 3). The effectiveness of the fractionation methods was checked using an artificial mixture of extracts from infected and uninfected cells; as expected, both dTMP synthetase and the inhibitor could be isolated from this mixture by the appropriate procedures provided the fractionations were started immediately after mixing (Tables 8 and 9, lines 2).

The behaviour of the dTMP hydroxymethylase in these fractionations was also checked (Tables 8 and 9). It did not precipitate in the same fraction as the inhibitor and unlike the latter was not inactivated by the use of solid ammonium sulphate.

The effect of the inhibitor on the substrates of dTMP synthetase.

The possibility that the inhibitor destroyed one or both of the substrates of dTMP synthetase, namely dUMP, HCHO and H_4PteGlu was additionally checked as follows. The substrates were not destroyed when incubated in turn with the inhibitor (Table 10). This result agreed with the results of the fractionation experiments and was also consistent with the fact that neither substrate of dTMP synthetase need be present

Table 10. Effect of inhibitor on the substrates of dTMP synthetase

Treatment	Activity of dTMP synthetase ΔA_{340}/ h
Normal assay for dTMP synthetase	0.53
Assay including sample of inhibitor, no incubation	0.47
Assay using incubated mixture of inhibitor with dUMP	0.52
Assay using incubated mixture of inhibitor with H_4PteGlu and HCHO	0.49

Ammonium sulphate fractions containing either inhibitor or dTMP synthetase were used. The inhibitor preparation was incubated for 15 min at 37° with each of the two substrates of dTMP synthetase individually and the incubation mixture then used as the sole source of the appropriate substrate in an assay for a constant amount of dTMP synthetase.

during the interaction *in vitro* of the enzyme with the inhibitor.

The time-course of the inhibition process and the effect of varying amounts of inhibitor.

The ammonium sulphate fractions containing dTMP synthetase and the inhibitor were used in the assay described in Chapter I (page 37) to follow the time-course of the inhibition process *in vitro*. The results (Fig. 14) showed that an initial fast rate of inhibition tailed off after approximately 5-10 min to a slower, apparently linear rate. The degree of inhibition at any chosen incubation time increased with the amount of inhibitor present but the increase was not linear.

The actual relationship for incubations of 5 min duration is shown in Fig. 15. In this experiment varying amounts of inhibitor were incubated with a fixed amount of dTMP synthetase. When large volumes of inhibitor were used, further increases in the amount of inhibitor had very little effect on the initial rate of inhibition, implying that the inhibitor was in excess. It was only when very small amounts of inhibitor were used and dTMP synthetase assumed to be in excess, that the initial inhibition rate increased approximately linearly with increases in the amount of inhibitor. Two 15 min incubations at the two lowest

Figure 14

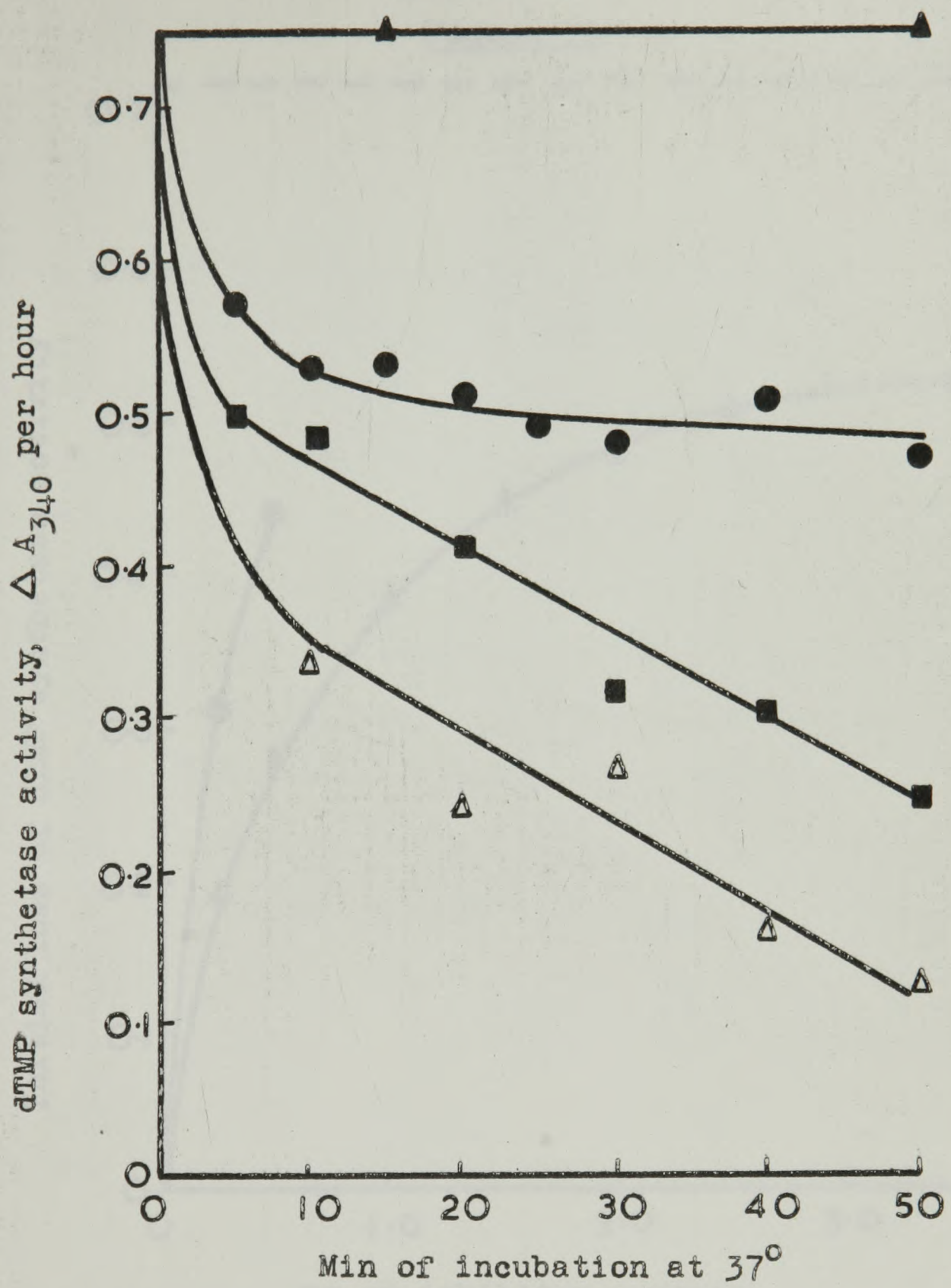


Fig. 14. The time-course of the inhibition process

Ammonium sulphate fractions containing either the inhibitor or dTMP synthetase were incubated together at 37° in a final volume of 0.5 ml of buffer D. The incubations were terminated by diluting and chilling the mixture and the residual dTMP synthetase was assayed

▲—▲ , ●—● , ■—■ and Δ—Δ were respectively nil, 0.3, 0.5, and 1.0 mg of inhibitor protein added per incubation to a constant amount of dTMP synthetase.

Figure 15

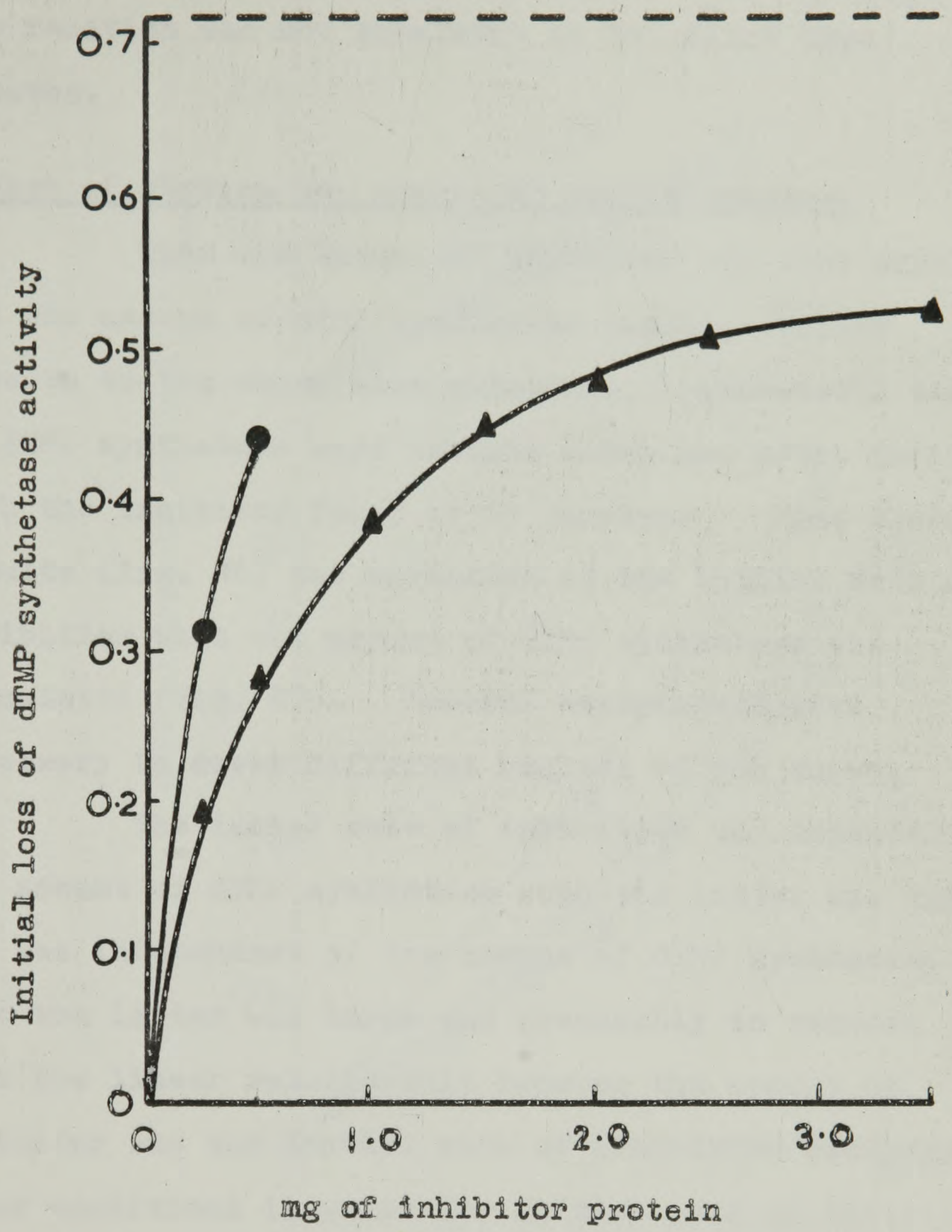


Fig. 15. Effect of varying the amount of inhibitor on the initial rate of inhibition of a constant amount of dTMP synthetase

Ammonium sulphate preparations of inhibitor and dTMP synthetase were incubated together in 0.5 ml buffer D for 5 min ▲——▲ and 15 min ●——● before chilling. The initial loss of enzyme activity was defined as $\Delta A_{340}/h$ for uninhibited enzyme minus $\Delta A_{340}/h$ for inhibited enzyme; - - - - - - - - shows the maximum possible loss.

rates of inhibition were also carried out to check that the reaction was not completed in the first five minutes.

Effect of varying the amount of dTMP synthetase

When the amount of inhibitor was kept constant and the amount of dTMP synthetase varied, similar results to the above were obtained. Increasing amounts of dTMP synthetase were assayed alone and after incubation with the inhibitor for 5 or 10 minutes. From these results (Fig. 16) the variation of the initial rate of inhibition with the amount of dTMP synthetase was calculated (Fig. 17). Several experiments were necessary to cover different regions of the curve.

The initial rate of inhibition was dependent on the amount of dTMP synthetase when the latter was small, but was independent of the amount of dTMP synthetase when the latter was large and presumably in excess. Thus the linear relationship between the amount of inhibitor and the initial rate of inhibition occurred under conditions in which the initial rate of inhibition was independent of the amount of dTMP synthetase. These results suggested that in order to assay the inhibitor quantitatively an initial rate of less than 30% inhibition in 5 min was required.

Stability of dTMP synthetase and the inhibitor.

A study of the individual stabilities of

Figure 16

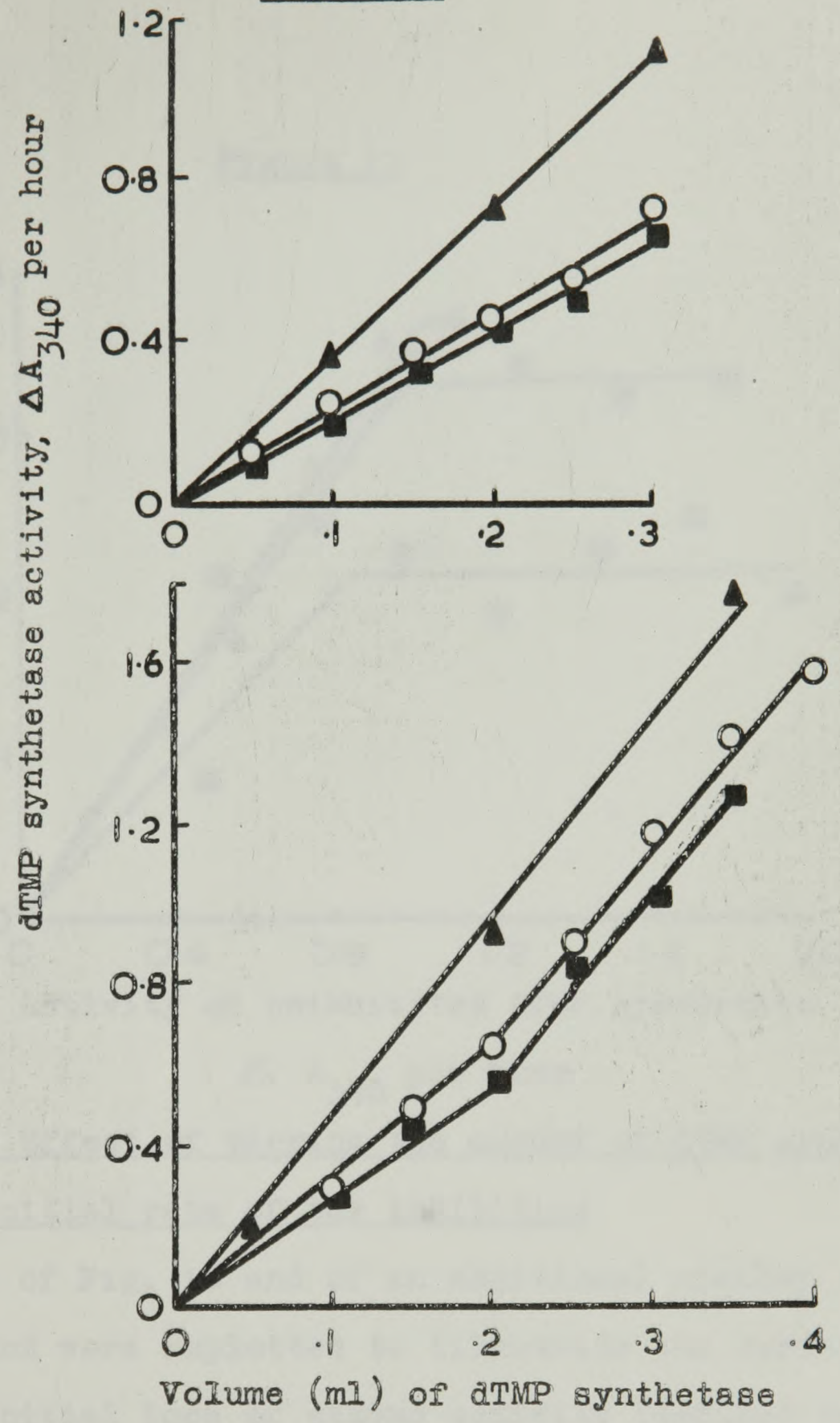


Fig. 16. Effect of varying the amount of dTMP synthetase on the initial rate of inhibition caused by a constant amount of inhibitor

Ammonium sulphate preparations of dTMP synthetase and the inhibitor were incubated together for 5min, o—o and 10 min, ■—■ at 37° in a final volume of 0.5 ml of buffer D. The incubations were terminated by chilling and the residual dTMP synthetase assayed. ▲—▲ shows the activity of the dTMP synthetase in the absence of the inhibitor.

Figure 17

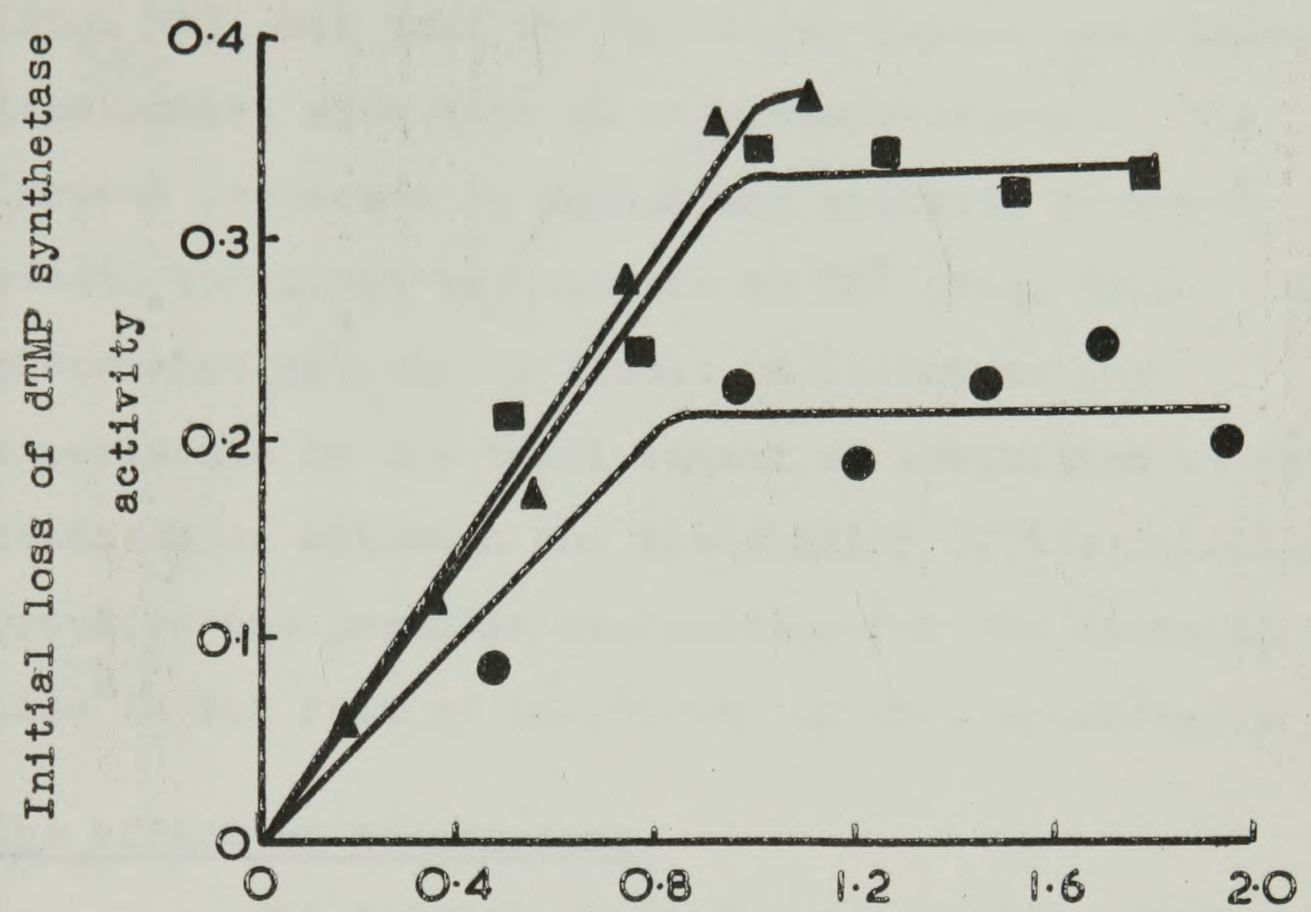


Fig. 17. Effect of varying the amount of dTMP synthetase on the initial rate of the inhibition

The data of Fig. 16 and of an additional similar experiment were replotted to illustrate the variation of the initial loss of enzyme activity (defined as $\Delta A_{340}/h$ for uninhibited dTMP synthetase minus $\Delta A_{340}/h$ for the inhibited enzyme after a 5 min incubation at 37°) with a fixed amount of inhibitor. ▲—▲ , ■—■ , and ●—● were respectively experiments in which 1.4, 1.28, and 0.9 mg of inhibitor protein were used per incubation.

dTMP synthetase and the inhibitor to the incubation conditions used to assay the latter showed that dTMP synthetase was stable at 37° for at least one hour (Fig. 14), but that the inhibitor became progressively less active with time at this temperature. The largest decreases in inhibitory activity occurred within the first ten minutes at 37° (Fig. 18). This phenomenon made it difficult to interpret any experiments on the total extent of inhibition under these conditions, although the instability of the inhibitor provided one possible explanation for the decrease with time in the rate of inhibition of dTMP synthetase.

The effect of temperature.

The initial rate of the inhibition process was also affected by the temperature of the incubation (Fig. 19). The rate of inactivation of dTMP synthetase was slower at 25° than at 37° and extremely slow at 0°. The reaction at 25°, like that at 37° was biphasic (compare Figs. 20 and 14). However the initial fast rate of inhibition lasted for 15 - 20 min at 25° instead of 5 - 10 min at 37°.

Attempts to reverse the inhibition

The results described in this chapter established many of the characteristics of the interaction between dTMP synthetase and the inhibitor but did not

Figure 18

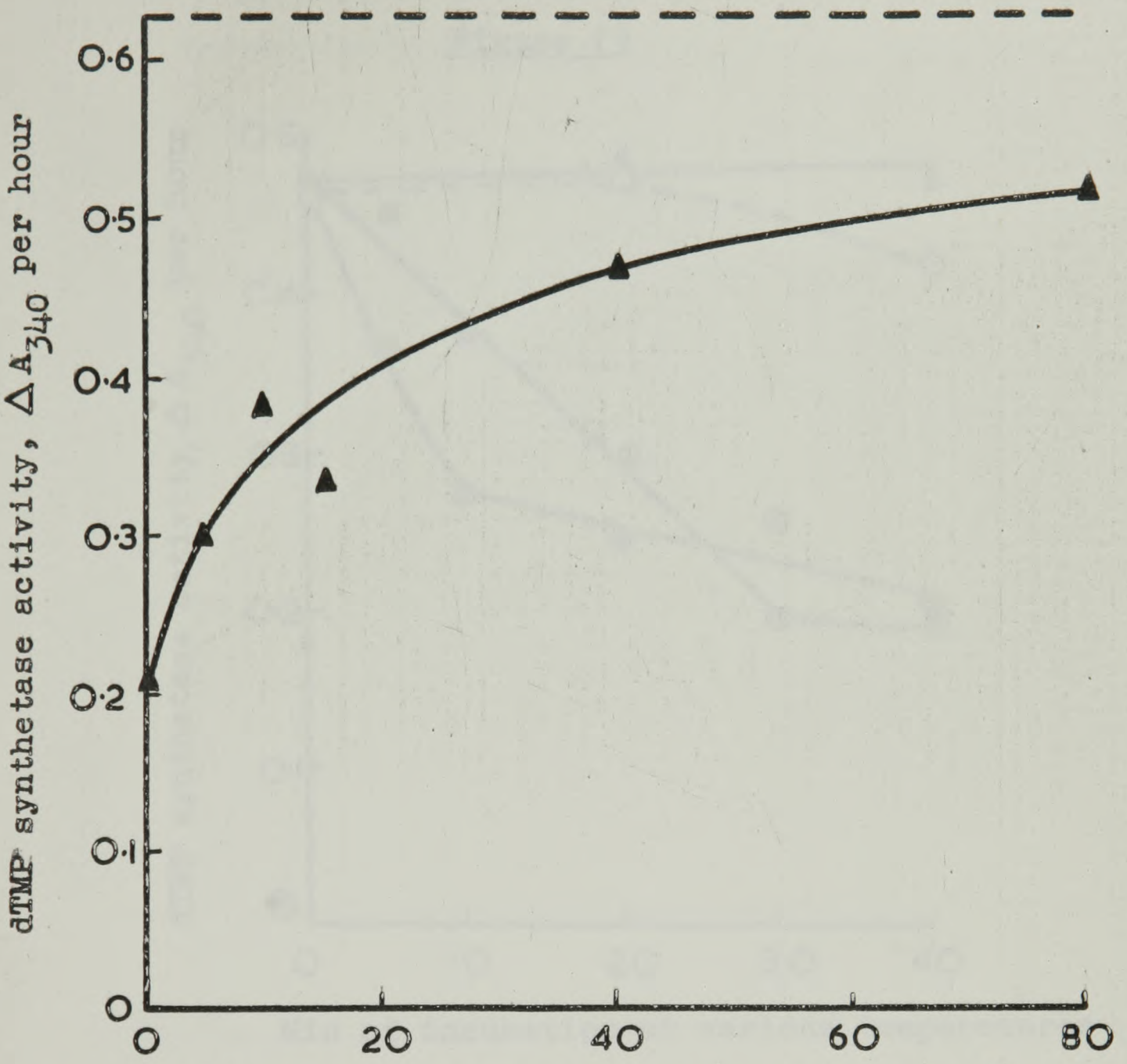


Fig. 18. Stability of the inhibitor of dTMP synthetase

The inhibitor preparation was incubated alone for various lengths of time in 0.4 ml of buffer D at 37° before incubating it with 0.1 ml of dTMP synthetase for 10 min at 37° and assaying the residual enzyme. - - - - - shows the corresponding activity of the uninhibited dTMP synthetase.

Figure 19

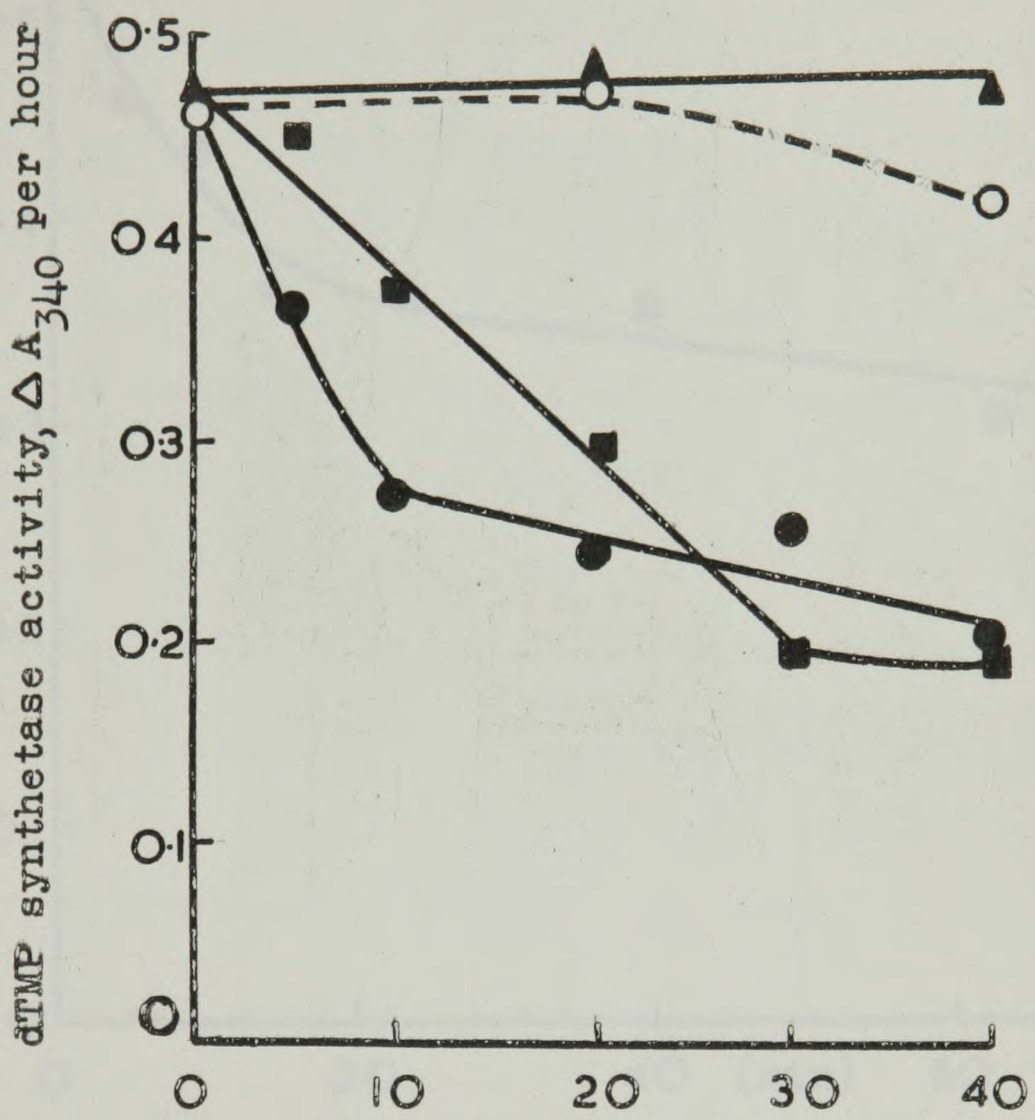


Fig. 19. Effect of varying the temperature of the incubation

Ammonium sulphate fractions of inhibitor and dTMP synthetase were incubated at the following temperatures

o—o 0°, ■—■ 25°, and ●—● 37° for different lengths

of time, chilled and the residual dTMP synthetase activity measured. ▲—▲ shows the activity of dTMP synthetase incubated at 37° in the absence of the inhibitor.

Figure 20

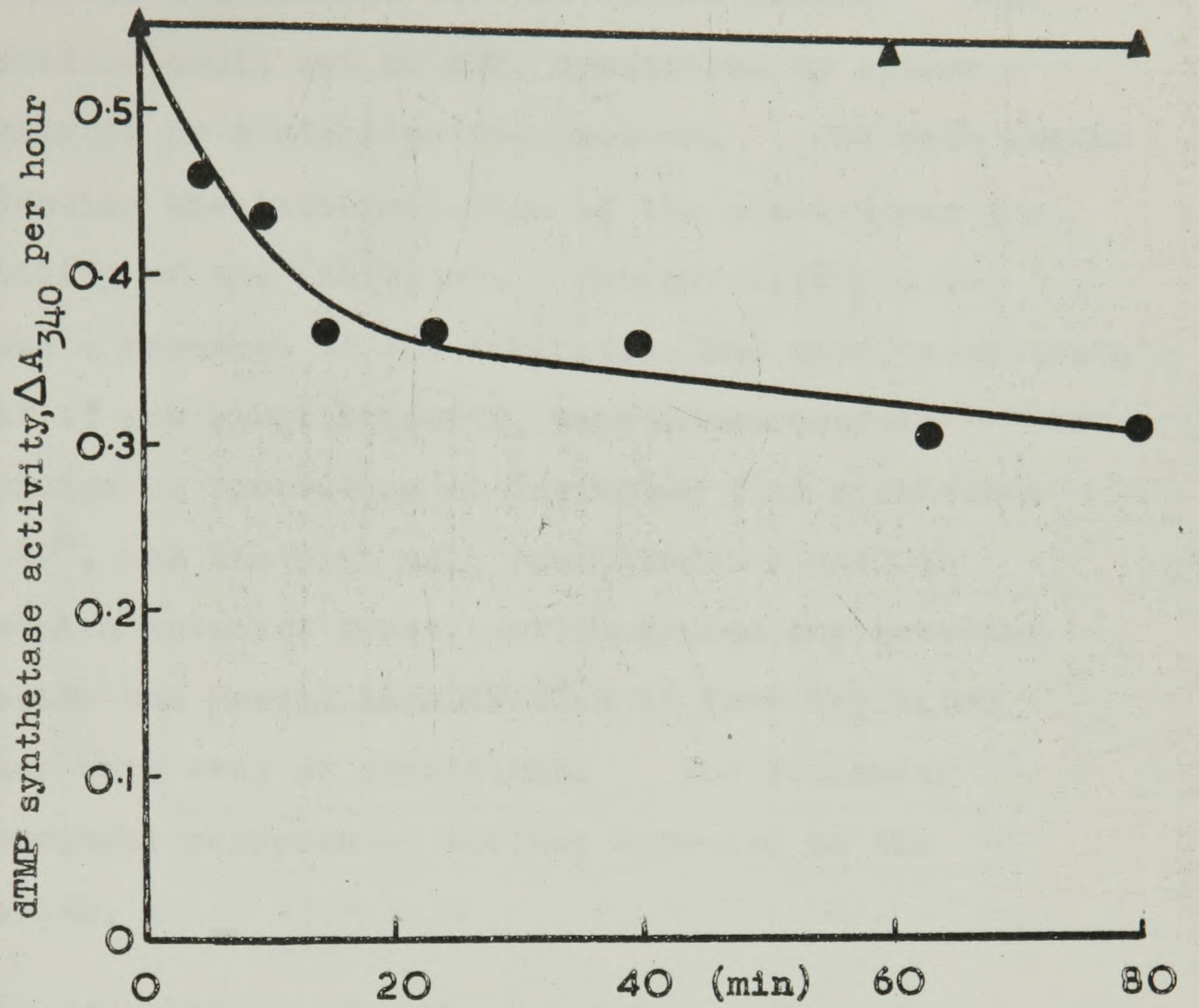


Fig. 20. Time-course of the inhibition reaction at 25°

●—● Ammonium sulphate fractions of the inhibitor and dTMP synthetase were incubated at 25° for different lengths of time, chilled and assayed.

▲—▲ dTMP synthetase was similarly incubated in the absence of the inhibitor.

establish a mechanism for the inactivation. The inhibitor could act on dTMP synthetase by either a catalytic or stoichiometric process. The main factor affecting the interpretation of the results was the lability of the inhibitor. Various attempts to cause a reversal of the inhibition and thus demonstrate that it was stoichiometric, were unsuccessful. Neither a prolonged incubation of inhibited dTMP synthetase at 37°, nor the high salt concentration used in ammonium sulphate fractionation caused any reversal despite the proven inactivation of free inhibitor under both sets of conditions. The following experiment represented another approach to the problem.

The effect of varying the conditions of sonic disruption

Some preliminary experiments showed that the initial rate of inactivation of dTMP synthetase (in 5 min) by the inhibitor *in vitro* was very low when the pH of the incubation buffer was 6 or 7 and increased with pH up to at least pH9 (Fig. 21). Therefore, if phage-infected cells were to be disrupted by sonication at the low pHs, the detection of large amounts of dTMP synthetase in the cell-free extract would imply either that the inhibition process was reversible, or that the inhibition of dTMP synthetase was entirely a post-sonication phenomenon. In the

Figure 21

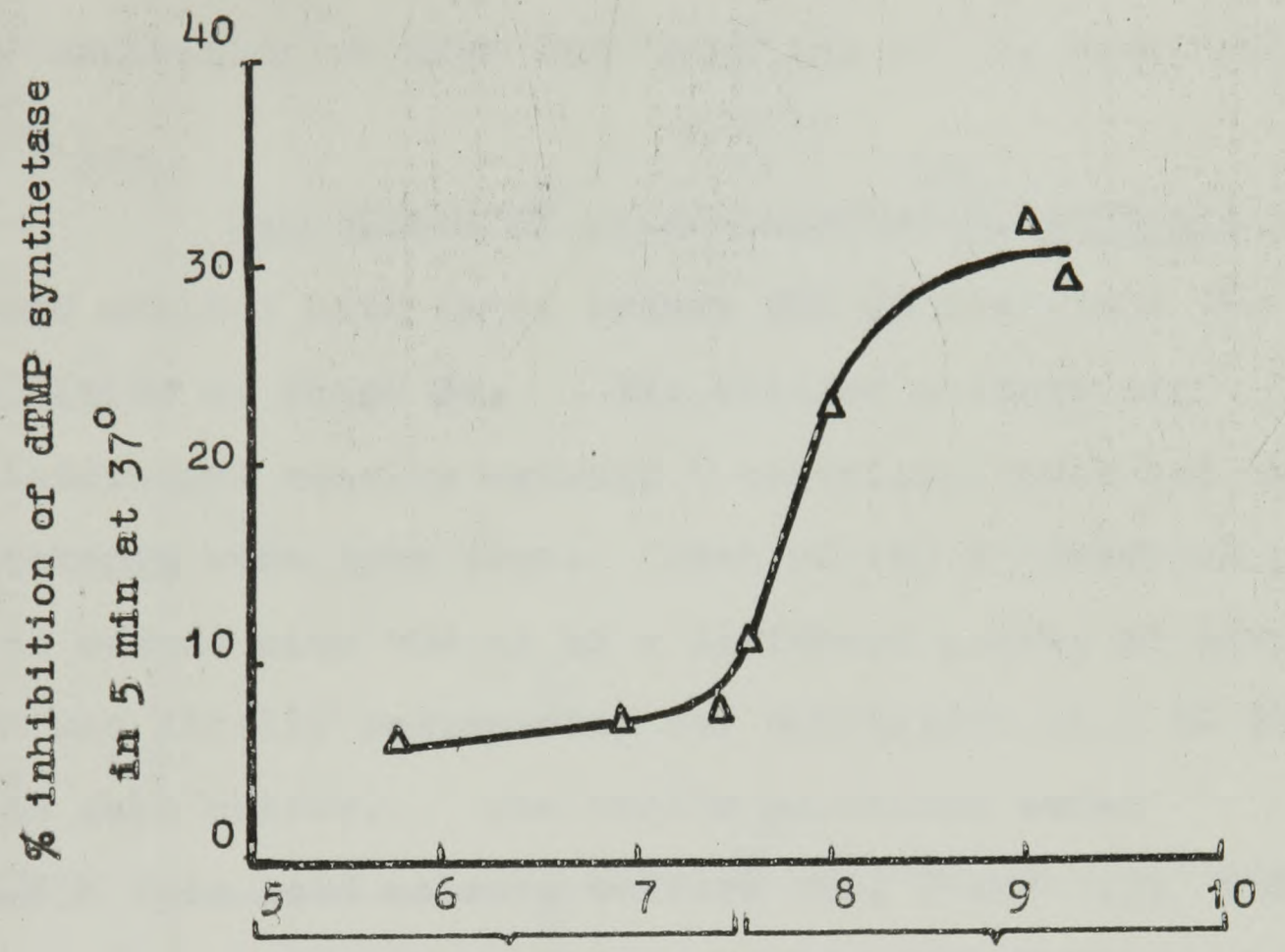


Fig. 21. Effect of varying the pH of the medium on the initial rate of inhibition

A small constant volume of inhibitor (0.01 ml) was equilibrated with 0.39 ml of each buffer at 0° for 2h; 0.1 ml of dTMP synthetase was then added and the mixture was incubated for 5 min at 37°, chilled and diluted with cold buffer A. The residual dTMP synthetase activity was then determined. Dialysed ammonium sulphate fractions of inhibitor and dTMP synthetase were used.

following experiment dTMP synthetase activity was determined in extracts from infected cells which had been disrupted by sonication at five different pHs in the range pH6 - pH9.

Two litres of phage-infected B. subtilis 3610 were chilled with 1l of frozen DMM 25 min after the addition of phage Øe. The chilled culture was distributed equally between 6 centrifuge pots and the infected bacteria were spun down. Each of the 6 bacterial pellets was washed with 100 ml of a different buffer of given pH and finally resuspended for sonication in 6 ml of the same buffer. The buffer solutions were: 0.05M Tris acid maleate buffers pH6, 7 and 7.5; buffer A pH 7.5; and 0.05M Tris/HCl buffers pH8 and 9. The infected cells were disrupted by sonication at 0^{o} for 4 min and the cell-free extracts were clarified by the usual centrifugations and assayed for dTMP synthetase activity.

The results are given in Fig. 22. There was considerably more dTMP synthetase activity in extracts from infected cells which had been disrupted at pHs 6 and 7 than in extracts from cells disrupted at the higher pHs. Moreover this activity was lost on raising the pH to 7.5 by overnight dialysis at 4^{o}, although no significant overnight decrease in dTMP synthetase activity occurred in the original undialysed

Figure 22

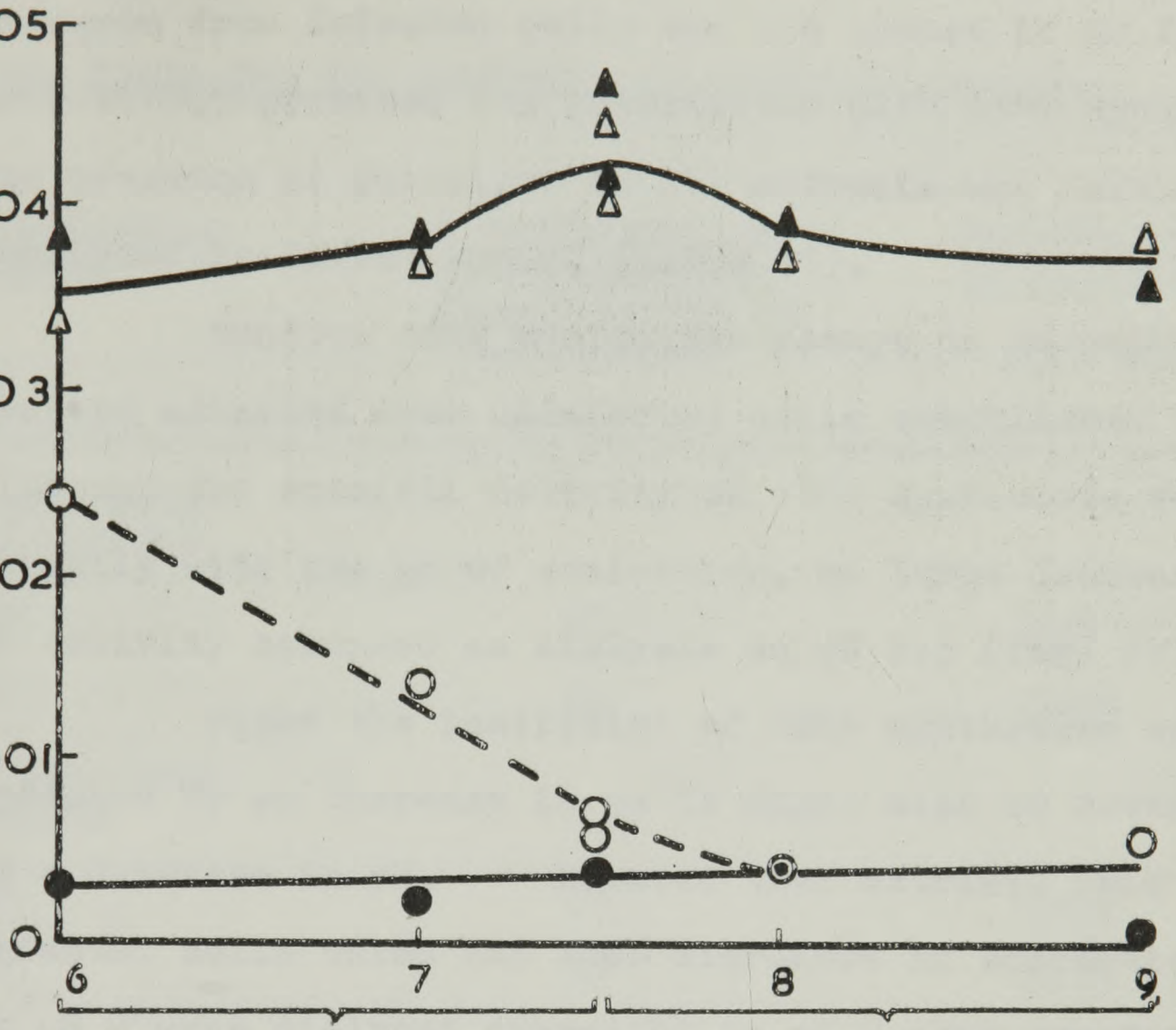


FIg. 22. The effect of varying the pH of sonication

The specific activity of dTMP synthetase was determined in extracts of cells which had been sonicated at different pHs.

Δ—Δ Uninfected B. subtilis 3610

o--o Phage Øe-infected B.subtilis 3610

The sonicated extracts at different pHs were dialysed overnight at 4° against buffer A to a common pH of 7.5 and the activity of dTMP synthetase re-determined.

▲—▲ Uninfected cells ●—● Infected cells

extracts. Thus the inhibitor was present in all extracts from infected cells and the change in pH from 6 or 7 to 7.5 promoted its interaction with dTMP synthetase. The presence of inhibitor in all extracts was further confirmed by direct assays (Table 11).

Control dTMP synthetase assays on identically-treated extracts from uninfected cells established that although the specific activity of dTMP synthetase varied slightly with the pH of sonication, no large decreases in activity occurred on dialysis to pH 7.5 (Fig. 22).

Since the inhibition of dTMP synthetase was promoted by an increase in pH it might also be reversed by a decrease in pH. However when extracts from infected cells which had been disrupted by sonication at pH 9 were dialysed overnight to pH 6, dTMP synthetase was not reactivated either by the change in pH or by re-sonicating the dialysed samples at pH 6 (Table 12).

When the activities of dTMP synthetase in extracts from uninfected and phage-infected cells which had been sonicated at pH 6 were compared, the latter extract contained at least 70% of the dTMP synthetase activity of the control. In a repeat experiment this figure was 100%. Therefore if dTMP synthetase was inhibited *in vivo* the inhibition was completely reversed by sonication at a pH which prevented the re-inactivation of dTMP synthetase by the inhibitor. This interpretation assumed that the inhibitor inactivated dTMP synthetase by

Table 11. Tests for the inhibiter in dialysed extracts

pH of sonication buffer	% inhibition of dTMP synthetase caused by 0.4 ml of extract	Protein concn. of extract mg / ml
0.05M Tris acid maleate buffers		
6	67	4.0
7	69	3.75
0.05M Tris / HCl buffers		
8	34	3.55
9	33	3.75

Cell-free extracts were prepared from phage Øe-infected B. subtilis 3610 by sonication at different pHs. The clarified extracts were then dialysed overnight against buffer D to a common pH of 7.5 and tested for the presence of inhibitor as follows; 0.4 ml of each extract was incubated with a dialysed ammonium sulphate sample of dTMP synthetase for 10 min at 37°. The incubation was terminated by dilution and chilling and the residual dTMP synthetase activity determined.

Table 12. Thymidylate synthetase activities in cell-free extracts

Treatment of cells	Specific activity of dTMP synthetase (μmoles of H_4PteGlu oxidised/h/mg protein)	
	Phage-infected *B. subtilis*	Uninfected *B. subtilis*
Experiment 1.		
Sonicated at pH 9	0.008	0.048
Sonicated at pH 9 ; extract dialysed to pH 6	0.008	0.033
Sonicated at pH 9 ; extract dialysed to pH 6 and resonicated for 1½ min	0.005	0.034
Experiment 2.		
Ground with alumina at pH 7.5	0.006	0.029

Experiment 1. A 600 ml culture of *B. subtilis* 3610 was chilled and harvested 25 min after the addition of phage Øe. The bacteria were resuspended for sonication in 15 ml of 0.05 M Tris / HCl buffer pH 9. The clarified sonicate was dialysed overnight against 0.05 M Tris acid maleate buffer pH 6 ; 4 ml of this dialysed extract was resonicated for 1½ min at 0°. A parallel uninfected culture of *B. subtilis* 3610 was similarly treated.

Experiment 2. A 400 ml culture of phage-infected *B. subtilis* 3610 was chilled and harvested 25 min after the addition of phage Øe. The bacteria were resuspended as if for sonication in 5 ml of buffer A (pH 7.5) but instead of sonicating the suspension it was homogenised at 0° with 0.5 gm of alumina in a power-driven teflon homogeniser. The extract was then clarified by the usual centrifugations. A parallel uninfected culture was similarly treated.

a reversible mechanism. However this experiment was also open to the interpretation that the inhibitor and dTMP synthetase were only brought together by cellular disruption, in which case no deductions about the mechanism of the inhibition process were possible.

Disruption by alumina grinding

The possibility that the inhibition of dTMP synthetase was a phenomenon limited entirely to extracts prepared by sonic disruption was excluded by preparing cell-free extracts by alumina grinding at pH 7.5 (Table 12). Although the specific activity in extracts prepared by this method from uninfected cells was 20 - 30% lower than in extracts prepared by sonication, the dTMP synthetase in extracts from phage-infected cells was still 80% inhibited by comparison.

Summary

Fractionation experiments showed that the inhibitor of dTMP synthetase did not act indirectly by interfering with the enzyme assay but acted directly on the enzyme. Ammonium sulphate fractions containing concentrated inhibitor and purified dTMP synthetase were used to show that the inhibition process was a time-dependent, temperature-dependent and pH-dependent reaction whose initial rate was a function of the relative amounts of inhibitor and dTMP synthetase.

Although no definite mechanism for the inhibition process could be inferred from the above results, one interpretation of the finding that extracts from infected cells disrupted at pH6 contained significant dTMP synthetase activity was that the inhibition process *in vivo* was reversible.

CHAPTER III The Purification of dTMP synthetase and the inhibitor.

Introduction

Purifications of dTMP synthetase and the inhibitor were undertaken for the following reasons.

1. To confirm the results obtained with the impure samples described in Chapter II.

2. In the hope that purification might improve the stability of the inhibitor.

3. With the ultimate intention of studying the interaction of the inhibitor with dTMP synthetase by means of a parameter other than the loss of enzymatic activity of the latter.

The purification of dTMP synthetase

Thymidylate synthetase has previously been extensively purified from calf thymus by Jenny and Greenberg (1963), from Streptococcus faecalis by Blakley and McDougall (1962), and from E. coli by Wahba and Friedkin (1962) and by Friedkin et al. (1962). The choice of procedure for the purification of dTMP synthetase from B. subtilis was influenced mainly by the methods used to purify the enzyme from E. coli. A summary of the purification adopted is given in Table 13. It reproducibly gave a 30 fold purification of dTMP synthetase.

Table 13. Purification of thymidylate synthetase

Step	Total volume ml	Total protein mg	Total units[x]	Specific activity units/mg protein	Purification
100 000 x g supernatant from streptomycin sulphate treatment	350	3100	265	0.085	Nil
Ammonium sulphate fraction after desalting on Sephadex G25	50	595	200	0.34	x 4
DEAE-cellulose pool after 'Aquacide' treatment	5.8	46	43	0.93	x 11
Sephadex G100 pool	6.4	8.6	21	2.45	x 29

[x] 1 enzyme unit catalyses the oxidation of 1 μmole of H_4PteGlu per hour at 25°

Preparation of the initial cell-free extract

In the course of some preliminary experiments it was observed that the specific activity of dTMP synthetase in cell-free extracts was related to the growth rate of the original bacterial culture and to the density at which the cells were harvested. A high initial specific activity of dTMP synthetase in the cell-free extract used for purification of the enzyme was achieved by growing B. subtilis 3610 on nutrient broth supplemented with glucose and by harvesting the cells in late logarithmic phase. The cells were disrupted by sonication; nucleic acids were precipitated with streptomycin sulphate; and the extract was clarified by centrifugation (Methods).

The initial specific activity of dTMP synthetase varied in different preparation from 0.075-0.10 units per mg protein and the average yield of enzyme units from 12l of culture was 200. Preparations in which the bacteria were stored at -15° before sonication yielded less dTMP synthetase of lower specific activity.

Ammonium sulphate fractionation

The crude extract was fractionated with ammonium sulphate as follows. 36.3g of solid ammonium sulphate were added per 100 ml of extract. The mixture was stirred at 4° for 20 min after all the solid had dissolved, and then clarified by centrifugation at

10 000xg for 10 min. A further 21.8g of solid ammonium sulphate per 100 ml of original extract was added to the supernatant and the precipitation and centrifugation were repeated. The second precipitate was resuspended in approximately 10 ml of buffer C and desalted by gel filtration.

The purification and recovery of dTMP synthetase in the above fractionation depended on the protein concentration of the crude bacterial extract. At the protein concentration routinely employed (6-9 mg/ml) the purification was four fold with a 70% recovery. At higher protein concentrations the purification increased but the recovery decreased; e.g. at 17.8 mg protein per ml the purification was seven fold with 50% recovery. The results of a small-scale pilot fractionation (Table 14) showed that dTMP synthetase was completely stable to ammonium sulphate. All the enzyme units in the unfractionated extract were accounted for in the various fractions.

To desalt the ammonium sulphate fraction containing dTMP synthetase the solution was layered onto a column of Sephadex G25, coarse, (50 x 1.6 cm), which had been equilibrated with buffer C. Thymidylate synthetase was eluted from the column using the same buffer. Tests for protein and sulphate ions showed that the excluded protein peak was usually separated

Table 14. Distribution of dTMP synthetase in ammonium sulphate fractions

	Total enzyme units	Total protein mg	Specific activity units/mg protein	Purification
Unfractionated extract	5.8	91	0.063	Nil
Cumulative gm ammonium sulphate added				
2.34	Nil	20	Nil	Nil
3.63	0.73	43	0.017	Nil
5.04	4.05	28	0.145	x 2.5
6.50	1.14	6	0.19	x 3
Total	5.92	97		

A 10 ml volume of cell-free extract was treated with solid ammonium sulphate at 4°. The precipitates were dissolved in buffer A and assayed for dTMP synthetase and protein.

from the retarded ammonium sulphate peak. Even when some overlapping occurred the contamination of protein by ammonium sulphate was not sufficient to affect the subsequent fractionation of dTMP synthetase on DEAE-cellulose.

Chromatography on DEAE-cellulose

The dTMP synthetase in the desalted ammonium sulphate fraction was adsorbed onto a column of DEAE-cellulose at pH 8.4 and further purified by elution using a concave gradient of NaCl in buffer B at pH 7.5 (Fig. 23). Both the linear salt gradient and the stepwise elution from DEAE-cellulose which were used in the purification of dTMP synthetase from E. coli were found to be unsatisfactory.

Thymidylate synthetase was eluted from the column as a main peak, followed by a smaller trailing peak (Fig. 23). Since only 60-70% of the enzyme was normally accounted for in these peaks the possibility that there was a third peak of activity was considered. Preliminary experiments gave no indication of this and it was concluded that dTMP synthetase was probably unstable to the conditions prevailing during the chromatography.

The fractions containing dTMP synthetase of highest specific activity were pooled. A three fold purification and 30% recovery were normal for this step.

Figure 23

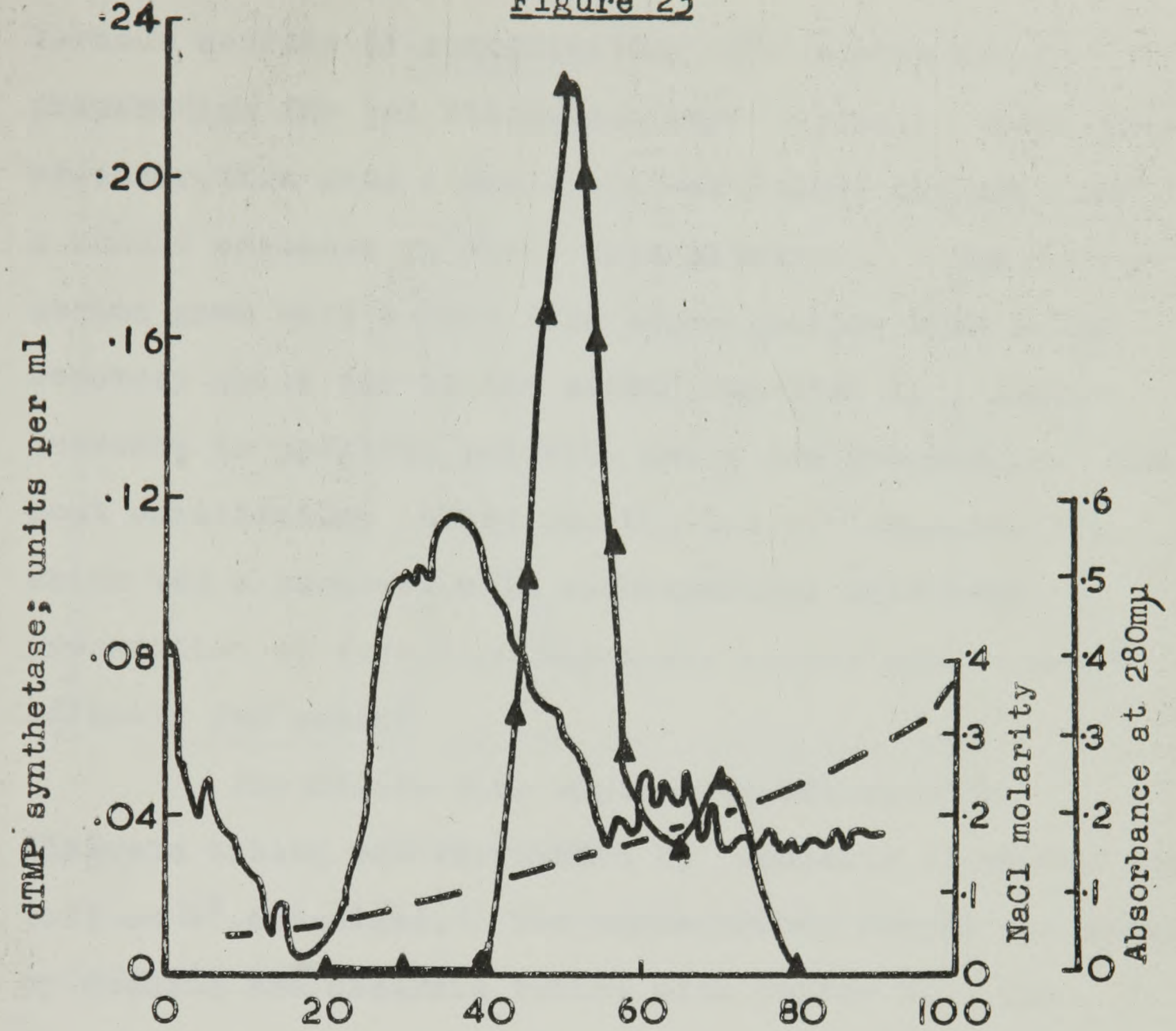


Fig. 23 Fractionation of dTMP synthetase on DEAE-cellulose at 4°.
The desalted ammonium sulphate fraction was run onto a column of DEAE-cellulose (25×1.6cm) which had been equilibrated with buffer C. The column was washedwith 50ml of buffer B containing 0.05M NaCl, and the effluent was discarded. The column was then subjected to a concave salt gradient as follows. The reservoir (diameter 7.1cm) contained 398ml of buffer B containing 0.4M NaCl. The mixing chamber (diameter 9.4cm) contained 627ml of buffer B containing 0.05M NaCl. One hundred 10ml fractions were collected at a rate of approx. 4-5 tubes per hour. The elution of protein was followed by the O.D. at 280mμ. The shape of the salt gradient was calculated from the formula given by Pontis and Blumson (1958).

– – – – salt gradient ——— protein

—▲——▲— dTMP synthetase

Various methods of concentrating dTMP synthetase in preparation for gel filtration were tested. They included re-adsorption onto a small DEAE-cellulose column; and a second ammonium sulphate precipitation. The former method gave only a four fold concentration with a low recovery while the latter method resulted in a large decrease in specific activity and a low recovery. The most satisfactory method was the use of 'Aquacide I', which was a water-soluble carboxymethyl cellulose preparation of very high molecular weight with a great affinity for water.

The dilute dTMP synthetase solution in dialysis tubing was surrounded by 'Aquacide I' powder and left at 4^{o} overnight. The concentrated enzyme was rescued by washing the dialysis tubing with buffer B. The final volume was thus reduced to as little as 5ml with no loss in specific activity and with a good recovery of dTMP synthetase activity.

Gel filtration on Sephadex G100.

Thymidylate synthetase was retarded by a column of Sephadex G100 and a further three to four fold purification of the concentrated enzyme was achieved by this method (Fig. 24). All the enzyme units put onto the column were accounted for in the eluate, although only the tubes containing dTMP synthetase of high specific activity were pooled, with a resultant

Figure 24

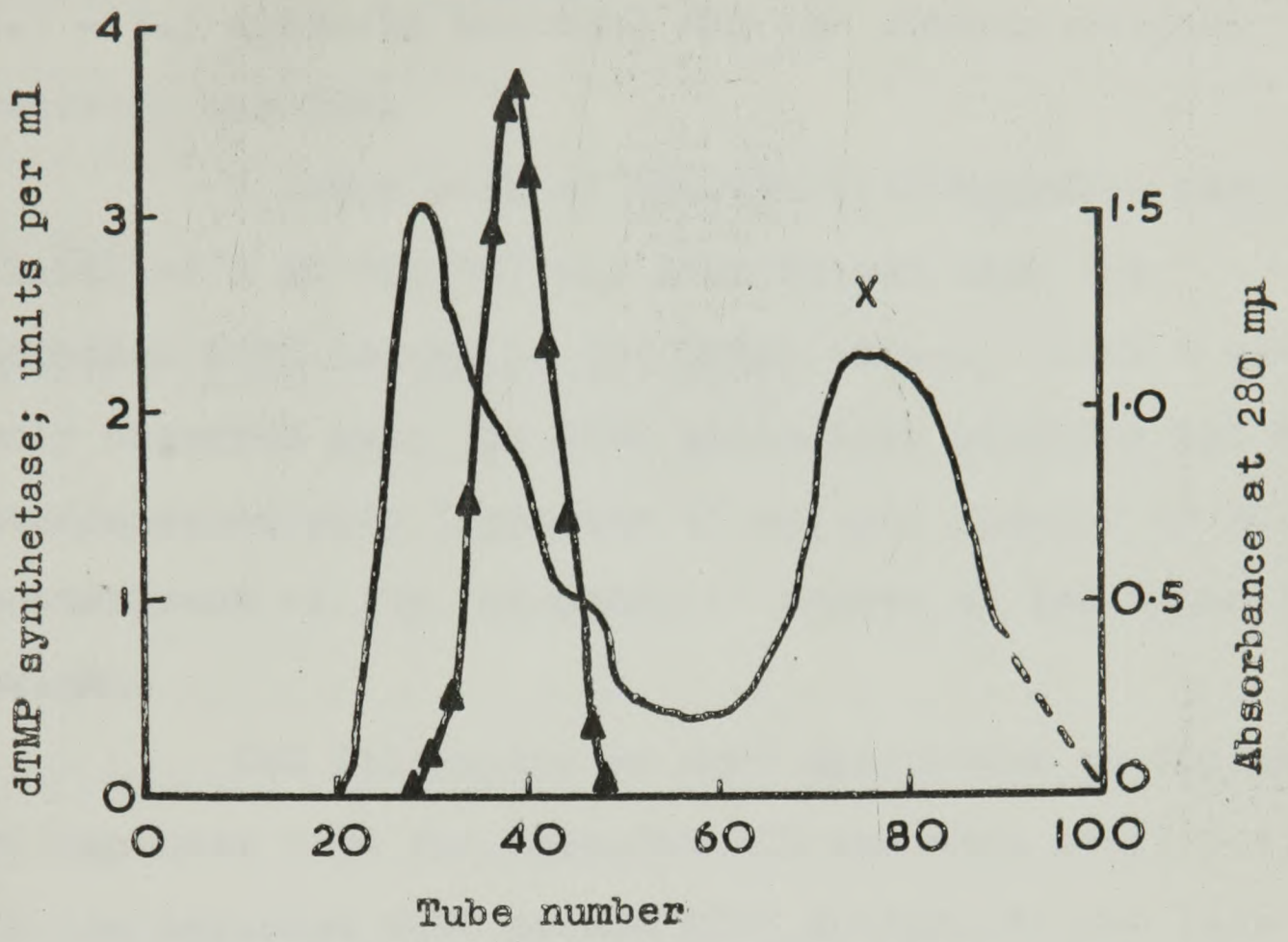


Fig. 24. Gel filtration of dTMP synthetase on Sephadex G100 at 4°

The concentrated DEAE-cellulose pool was layered onto a column of Sephadex G100 (50x1.6 cm) which had been equilibrated with buffer B. The enzyme was displaced downward with buffer B. The effluent fluid was collected in 1 ml fractions at a rate of approximately 20 ml per hour. The elution of protein was followed by the absorbance at 280 mμ.

▲——▲ dTMP synthetase

——— protein

recovery of 50-60%. The final specific activity was between 1.5 - 3.5 units/mg protein, and the average overall recovery was 13%.

A large peak of ultraviolet-absorbing material (labelled x in Fig 24) was also eluted from the Sephadex G100 column in the final tubes. Peak X was only observed when the dTMP synthetase solution had been concentrated with 'Aquacide I' and was assumed to be a contaminant of the 'Aquacide I' powder of low molecular weight.

Gel filtration of dTMP synthetase on columns of Sephadex G200 and Sephadex G75 was also attempted. On the Sephadex G200 column dTMP synthetase was less well separated from contaminating protein and a greater dilution of enzyme occurred when compared with the fractionation on Sephadex G100. Thymidylate synthetase was slightly retarded by Sephadex G75 and the purification thus obtained was only marginally less than that achieved using Sephadex G100. In this repect the dTMP synthetase of *B. subtilis* was similar to the *E. coli* enzyme which was purified three fold on Sephadex G75 (Friedkin *et.al.*, 1962).

The purified dTMP synthetase was stored at -15° until required. No loss in activity had occurred when the enzyme was re-thawed provided the process was not repeated. The enzyme was reasonably stable at 0°

but the stability of different preparation at 37° varied. The less pure preparations with higher protein concentration were most stable. The pure preparations were used in the purification of the inhibitor described below.

Polyacrylamide gel electrophoresis

The heterogeneity of samples of purified dTMP synthetase was examined by means of polyacrylamide gel electrophoresis (Methods). At least six different protein bands were separated (Fig. 25). This was a considerable improvement on the number of bands given by the initial crude extract, but showed that the purification of dTMP synthetase was incomplete.

The purification of the inhibitor of dTMP synthetase

The purification of the inhibitor of dTMP synthetase was considerably complicated by its instability and by the consequent need for a simple, rapid method. The procedure summarised in Table 15 was successfully repeated five times. The results quoted were taken mainly from the two most active preparations because considerable variation in the activity of the inhibitor was observed in different preparations.

The quantitative assay for the inhibitor.

A quantitative assay for the inhibitor was required in order to estimate the purification and

Figure 25

Polyacrylamide gel electrophoresis of dTMP synthetase preparations

Samples containing 50-100 µg of protein (in buffers A or B at pH 7.5) were made up to a concentration of 20% sucrose and pipetted into prepared electrophoresis tubes *in situ* in the electrophoresis apparatus. The reservoir buffer was carefully layered onto the samples until the tubes were full. The level of the buffer in the upper reservoir was then so adjusted that the surface was above the tops of the tubes. A small volume of bromo-phenol blue was added to the upper reservoir and the electrophoretic separation was run at 1 mA / tube until the blue marker ring had descended approx. ⅔ of the gel.

Tube A = 100 000 x g initial supernatant (100 µg protein)

Tube B = 100 000 x g initial supernatant (50 µg protein)

Tubes C = Sephadex G100 pool (50 µg protein)

(The gels were stained and stored as described in Methods).

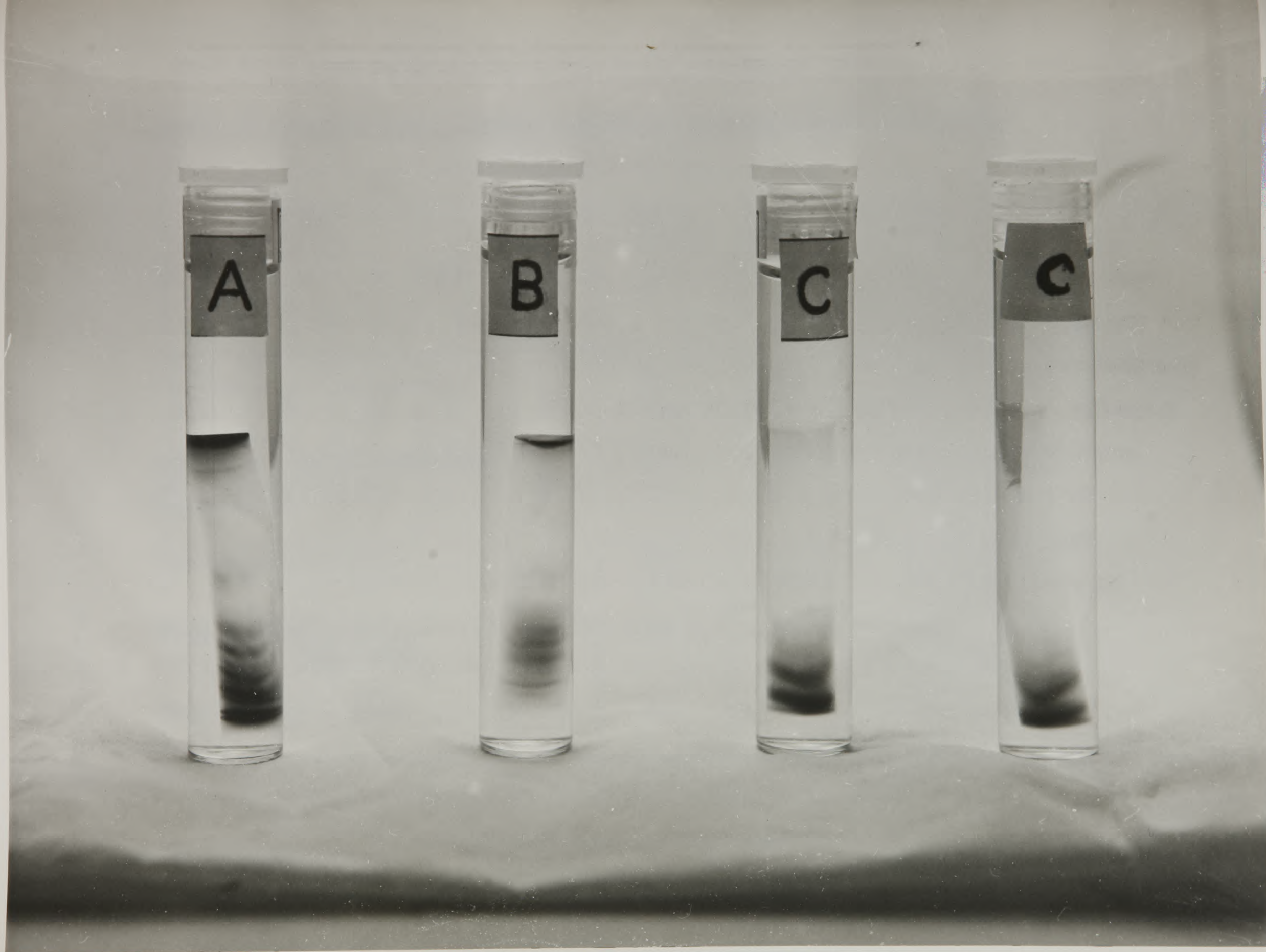

A
B
C
C

Table 15. Purification of the inhibitor of thymidylate synthetase

Step	Expt.	Total volume ml	Total protein mg	Total units[x]	Specific activity Units/mg protein	Purification
100 000 x g supernatant	1	104	850	495	0.58	Nil
	2	84	1250	440	0.34	Nil
Ammonium sulphate fraction after desalting on Sephadex G25	1	24	26.4	79	3.41	x 6
	2	25	137	16.5	0.12	?
DEAE-cellulose pool	1	80	5.2	74	14.2	x 24
	2	90	10.8	40.5	3.8	x 11
Sucrose-concentrated inhibitor	2	18	7	9.3	1.3	x 4
(Sephadex G100 pool	1	10	0.38	1.6	4.2	x 7)

[x]For definition of one unit of inhibitor see text

recovery at each stage of the fractionation. The relationship between the initial rate of inhibition and the amount of inhibitor was selected as the basis of the assay, and it was convenient to choose conditions under which the rate of inhibition was dependent only on the amount of inhibitor present and independent of the activity of the dTMP synthetase used in the assay. It was suggested on page 51 that such conditions prevailed approximately at low initial rates of inhibition when dTMP synthetase was in excess. The inhibitor was therefore assayed as follows.

A range of volumes of a sample containing the inhibitor were incubated with purified dTMP synthetase for 10 min at 37° in a final volume of 0.6 ml of buffer C, pH 8.4, (0.5 ml in purification 2 only). This pH was selected because the inhibitor was more active at pH 8.4 than at the pH of 7.5 employed during the experiments described in Chapters I and II. The results were plotted to show the number of units of dTMP synthetase inactivated by different amounts of inhibitor protein. Fig. 26 shows the types of graphs thus obtained. The specific activity of the inhibitor was calculated by extrapolating from within the linear region of each assay the number of units of dTMP synthetase destroyed by 1 mg of inhibitor protein. One unit of inhibitor thus inactivated one unit of dTMP synthetase in 10 min under the conditions of the assay.

When monitoring the inhibitory activity of

Figure 26

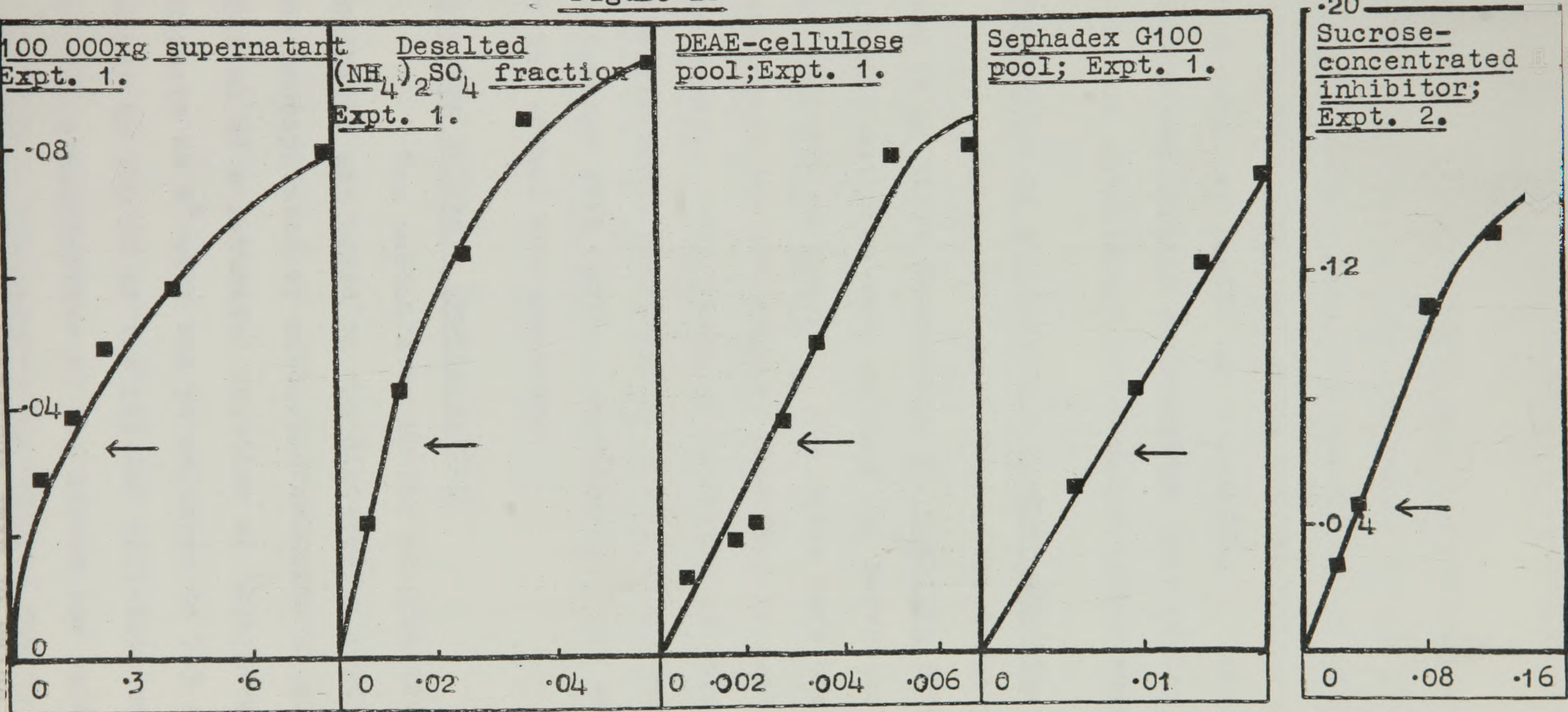


Fig. 26.Illustration of the assays for the inhibitor at several stages of purification

The arrows mark the position of 20% inhibition in each experiment.

The specific activities of dTMP synthetase preparations used were as follows:
Expt. 1 assays, 1.5 units per mg protein
Expt. 2 assays, 4.8 units per mg protein.

individual effluent tubes from column chromatography it was quicker and more convenient to use the original semiquantitative assay for the inhibitor to detect a peak of inhibitory activity, because only a single incubation per sample was necessary. Tubes containing significant amounts of inhibitor were pooled and the pool then assayed using the quantitative assay.

Preparation of the initial cell-free extract.

The initial cell-free extract was prepared from 6l of phage Øe-infected B. subtilis 3610. The infected cultures were chilled and harvested 25 min after the addition of phage. The cells were broken by sonication and the extract clarified by centrifugation (Methods). The yield of inhibitor at this stage in five different preparations varied by a factor of ten, indicating that further investigation and standardisation of the method was necessary.

Ammonium sulphate fractionation

The second step in the purification of the inhibitor was based on the discovery (Table 4) that it was precipitated by saturated ammonium sulphate solution. 66.6 ml of a saturated solution of 'enzyme-grade' ammonium sulphate at 4° with the pH adjusted to 7.5 was added slowly to 100 ml of the initial cell-free extract. The protein concentration of the latter was approximately 9 mg/ml. The mixture was stirred for 30 min and then

centrifuged at 10 000 xg for 10 min. The precipitate was resuspended in approximately 8 ml of buffer C and desalted on a column of Sephadex G25 as described for the desalting of dTMP synthetase.

Chromatography on DEAE-cellulose

The desalted ammonium sulphate fraction was adsorbed onto a column of DEAE-cellulose at 4^{o}, pH 8.4, (Fig. 27). The inhibitor was eluted as a single peak of activity using a linear salt gradient. The fractions containing the inhibitor were pooled. The dilute inhibitor was concentrated approximately four fold by the method used to concentrate dTMP synthetase but replacing the 'Aquacide I' powder with dry sucrose. The sucrose was chosen because of its ability to stabilise the inhibitor and because concentration with 'Aquacide I' was believed to introduce unknown impurities of low molecular weight. The sucrose and NaCl were removed from the concentrated inhibitor by dialysis at 4^{o} for 24h against one litre of buffer C with three changes of medium. Some loss in the specific activity of the inhibitor occurred during the concentration stage.

The sucrose-concentrated inhibitor was used to study the interaction of the purified inhibitor and dTMP synthetase. It was usual to use the inhibitor as soon as it was prepared since storage of

Figure 27

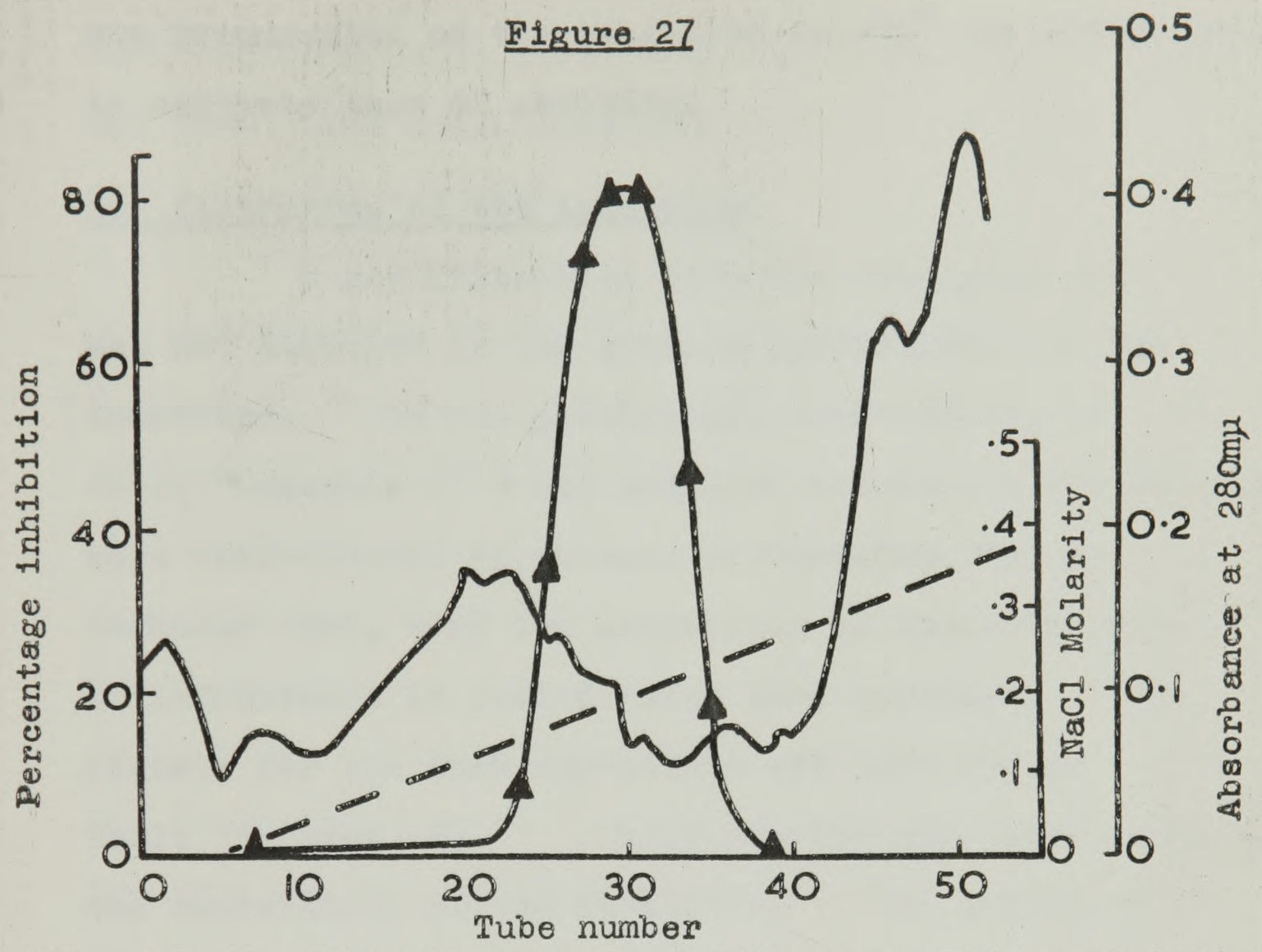


Fig. 27. Fractionation of the inhibitor on DEAE-cellulose at 4°.

The desalted ammonium sulphate fraction was run onto a column of DEAE-cellulose (25 x 1.6cm) which had been equilibrated with buffer C. The column was washed with 20ml of buffer C, discarding the effluent, and then subjected to a linear salt gradient as follows. The reservoir contained 300ml of buffer C containing 0.45M NaCl and the mixing chamber held 300ml of buffer C. 10ml fractions were collected at a rate of approx. 2-3 tubes per hour. The elution of protein was followed by the absorbance at 280mμ. To detect the elution of the inhibitor 0.2ml samples were incubated with dTMP synthetase (specific activity 0.6 units per mg protein) in a final volume of 0.6ml buffer C for 10min at 37°. The residual dTMP synthetase was assayed and the percentage inhibition calculated.

– – – –salt gradient ▲—▲ inhibitor

———— protein

one preparation of the inhibitor at -15° for a week resulted in complete loss of activity.

Gel filtration of the inhibitor.

A gel filtration step was attempted but was not included in the routine purification of the inhibitor. In two preliminsary experiments, in which 'Aquacide I' - concentrated samples of the inhibitor were fractionated on columns of Sephadex G100 and Sephadex G200, very low recoveries of inhibitor and no improvement in purification were obtained. The figures for one such experiment are included in Table 15 (Expt. 2). These results were attributed to the instability of the inhibitor. The inhibitor was retarded by the column of Sephadex G100 to approximately the same extent as dTMP synthetase as judged by the relative position of each to the excluded protein front and to the contaminating 'Aquacide I' peak. In agreement with this result the inhibitor was considerably retarded by the Sephadex G200 column.

Comments on the purification procedure.

The purification achieved at each stage in two different experiments is shown in Table 15. The specific activity of the inhibitor in the dilute DEAE-cellulose pool was invariably higher than the specific activity of the initial crude extract, and apparent

purifications of up to 20 fold were attained. However the true purification was likely to be somewhat lower for the following reasons.

1. The linearity of the assay increased with the purification of the inhibitor (Fig 26). The inhibition caused by the inhibitor in the dilute DEAE-cellulose pool and in the sucrose-concentrated DEAE-cellulose pool was linear with volume up to a rate of approximately 40% inhibition in 10 min. (This effect was also observed with the pooled inhibitor from the preliminary gel filtration experiment on Sephadex G100). The inhibition caused by the desalted ammonium sulphate fraction was only linear up to a rate of approximately 20% inhibition in 10 min while the inhibition caused by the crude extract had essentially no linear region.

2. Apparent recoveries of greater than 100% in the purification step involving DEAE-cellulose chromatography were observed in two of the five purifications (e.g. Expt. 2, Table 15).

A possible simple explanation for these results was that in the crude fractions the presence of other proteins interfered with the assay for the inhibitor.

Characteristics of the interaction of purified dTMP synthetase with the partially purified inhibitor.

The interaction of the purified preparation of dTMP synthetase and the sucrose-concentrated inhibitor was investigated in some preliminary experiments at pH 8.4. The effect of varying amounts of inhibitor on the inhibition obtained was described in the section on the quantitative assay for the inhibitor.

The time-course and temperature-dependence of the interaction is shown in Fig. 28. No inhibition occurred in this experiment during a 40 min incubation at 0^{o}, although in other experiments a small amount of inactivation occurred at this temperature. However at 37^{o} an initial fast reaction was followed by a slower one. Although the inhibitor preparation used was stable to incubation at 37^{o} (Fig 29) it was not possible to determine whether the interaction was proceeding to an equilibrium position because the purified dTMP synthetase itself was unstable to incubation at 37^{o}. This instability decreased as the protein concentration of the incubation mixture increased and was superimposed on the inactivation of dTMP synthetase by the inhibitor.

These results, although preliminary and incomplete, re-inforced some of the results which were obtained using ammonium sulphate fractions of the inhibitor and of dTMP synthetase. at pH 7.5.

Figure 28

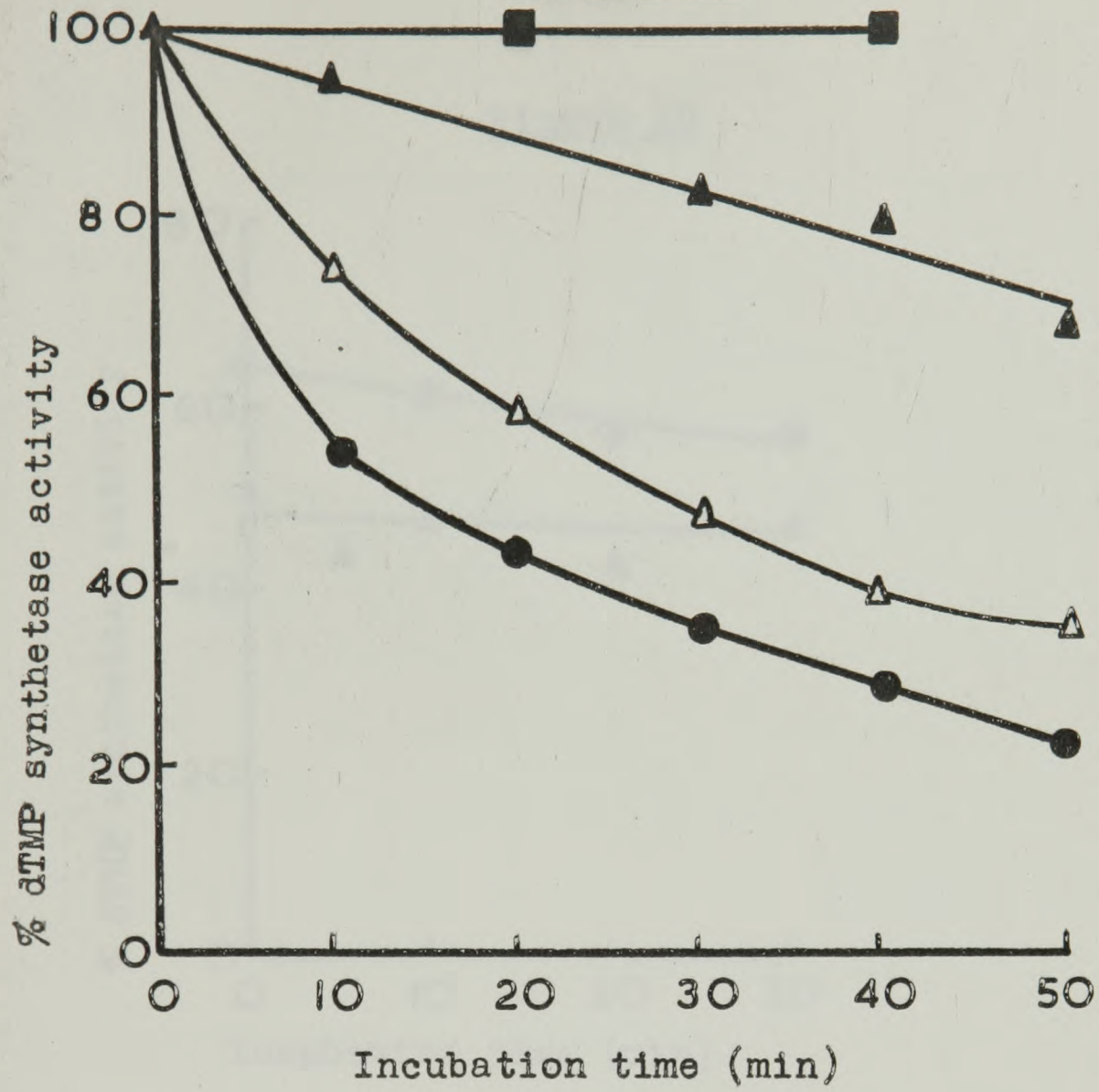


Fig. 28. The time-course and temperature-dependence of the inhibition reaction using purified components

The sucrose-concentrated sample of the inhibitor (purification 2) was incubated with 0.21 units of dTMP synthetase (specific activity 4.8 units per mg protein) in a final volume of 0.5ml of buffer C for varying lengths of time. The mixture was diluted and chilled and the residual dTMP synthetase assayed.

■——■ 0.04mg inhibitor protein incubated at 0° with dTMP synthetase
△——△ 0.04mg inhibitor protein incubated at 37° with dTMP synthetase
●——● 0.08mg inhibitor protein incubated at 37° with dTMP synthetase
▲——▲ dTMP synthetase incubated alone

Figure 29

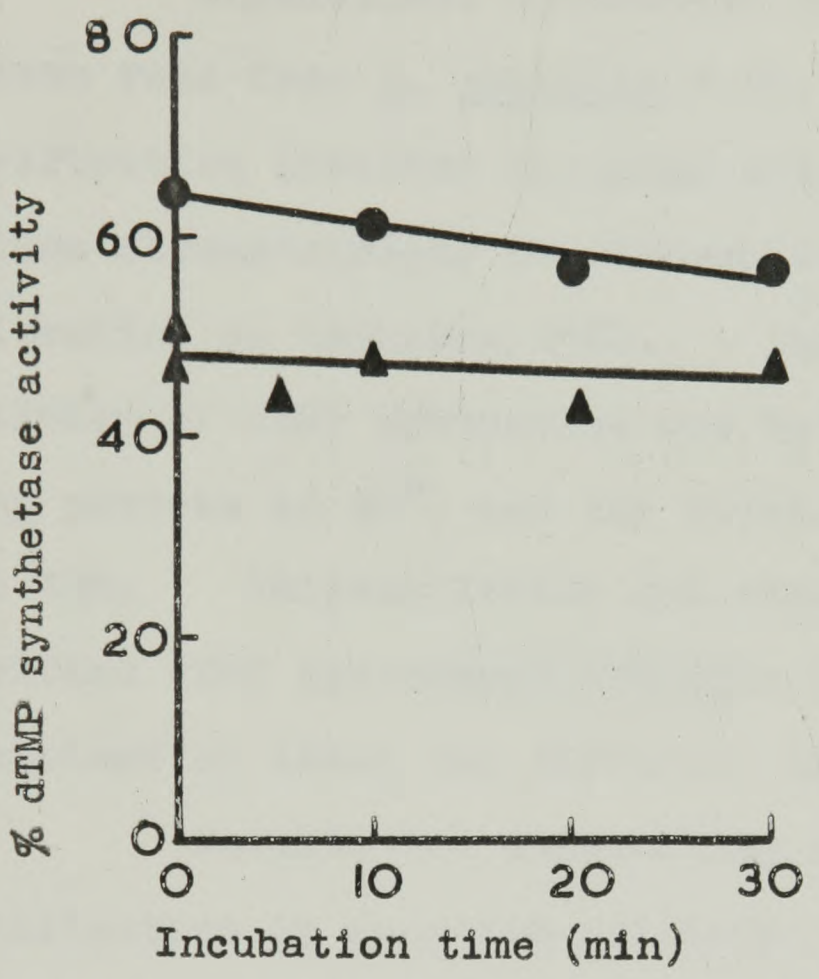


Fig.29. Stability of the purified inhibitor

Two different volumes of sucrose-concentrated inhibitor were incubated alone at 37^{0} in a final volume of 0.4ml of buffer C (pH 8.4) for different lengths of time. The residual activity of the inhibitor was then determined by a 10min incubation with dTMP synthetase as described in Fig. 28.

●——● 0.04mg inhibitor protein

▲——▲ 0.08mg inhibitor protein

Summary

Thymidylate synthetase was purified approximately thirty fold from B. subtilis 3610. The three stage purification involved ammonium sulphate fractionation, column chromatography on DEAE-cellulose and gel filtration on Sephadex G100. The final specific activity of dTMP synthetase was between 1.5 - 3.5 units / mg protein at 25°, and the average overall recovery was 13%. Polyacrylamide gel electrophoresis of the purified dTMP synthetase solution showed that it contained at least six different protein species.

Despite the instability of the inhibitor some purification by ammonium sulphate fractionation and chromatography on DEAE-cellulose was achieved. A quantitative assay for the inhibitor was devised. The interaction of purified samples of the inhibitor and dTMP synthetase appeared to be similar to the interaction of the ammonium sulphate fractions, although the stability of the inhibitor had increased and that of dTMP synthetase had decreased by comparison with the less pure samples.

DISCUSSION

DISCUSSION

The purpose of this work was to investigate various aspects of the control of thymine biosynthesis during phage infection of B. subtilis, particularly the phage Øe-induced loss of dTMP synthetase. The results are discussed with regard to the following problems.

Section 1. The cause of the inhibition of dTMP synthetase.

Section 2. The mechanism of the inhibition process.

Section 3. The cellular origin and physiological role of the inhibitor.

Section 1. The cause of the inhibition of dTMP synthetase. The properties and assay of an inhibitor of dTMP synthetase.

The results clearly showed that cell-free extracts of phage-infected B. subtilis contained an inhibitor of dTMP synthetase whose appearance accompanied the disappearance of the enzyme. An important feature of the interaction of dTMP synthetase with the inhibitor in vitro was the slow rate and temperature-dependence of the process. A reproducible inhibition of dTMP synthetase in a mixture of extracts from infected and uninfected bacteria was observed only if the mixture was preincubated at 37°. This finding explained why Roscoe (1965) was unable to detect any

inhibitor of dTMP synthetase by simply mixing extracts.

The inhibitor had many of the properties of a protein. It was heat-labile, sensitive to trypsin, and of macromolecular proportions as shown by its inability to pass through dialysis tubing and by its exclusion from Sephadex G25. A characteristic of the inhibitor which it shares with many other proteins and enzymes was its instability both to storage and to the incubation conditions necessary for its assay. During the investigation of the interaction of ammonium sulphate fractions containing the inhibitor and dTMP synthetase the inhibitor was stabilised to storage by the presence of 10% glycerol. Samples could then be kept at 4° for at least five days, or frozen and thawed with the retention of about 70% of the original activity. Glycerol is believed to stabilise hydrophobic groups (Tanford, 1964). When buffer solutions containing glycerol were not employed it was advisable to use freshly prepared extracts, and during the purification of the inhibitor the emphasis was on speed.

Despite the instability of the inhibitor it was partially purified from the initial cell-free extract by precipitation with ammonium sulphate followed by column chromatography on DEAE-cellulose. Attempts to purify the inhibitor further by gel filtration resulted in large losses of activity. The recoveries

from the DEAE-cellulose column in all purifications were apparently 100% or greater. A simple explanation for this phenomenon is that the assays for the inhibitor in the crude extract were inaccurate. This possibility is discussed below. An alternative and less likely explanation is that a complex of inhibitor and dTMP synthetase dissociated during chromatography to release the free components. This presupposes that the hypothetical complex of inhibitor and enzyme precipitates at least partially in the same ammonium sulphate fraction as the inhibitor. It also assumes that the dissociated dTMP synthetase is enzymically inactive, because no peak of enzyme was eluted from the DEAE-cellulose column during the fractionation of the inhibitor, although the salt gradient in one experiment reached 0.5M NaCl.

The inhibitor was assayed by using the relationship between the initial inhibition (attained in a 5 or 10 minute incubation with dTMP synthetase) and the amount of inhibitor added. A similarly-based assay was used by Mecke et al. (1966) to estimate a glutamine synthetase inactivating factor in E. coli. The relationship was approximately linear, and the assay therefore quantitative, only at low initial rates of inhibition in the presence of a large excess of the purified dTMP synthetase. The extent of the

linear region also increased progressively during the three purification stages of the inhibitor, so that although the assay was most reliable as a qualitative means of detecting the inhibitor, it probably became quantitative only after some initial purification.

During assays for the inhibitor in the crude extract the presence of a high concentration of contaminating proteins might affect both the interaction of the inhibitor with the purified dTMP synthetase, or the subsequent assay for the residual enzyme. The assay for dTMP synthetase was known to be somewhat unsatisfactory at high concentrations of non-specific protein (Methods). The increasing stability of the inhibitor during its purification could also affect the accuracy of its assay. In future it might be advisable to modify the assay to minimize the latter possibility by using a shorter incubation time (5 min) at a lower temperature (25°). Such a modified assay was not chosen in the present work since it would be less sensitive to small amounts of inhibitor.

The final confirmation that the inhibitor is a protein, and an estimate of its molecular weight must await an investigation of a purified sample.

The site of action of the inhibitor.

The protein-containing material which caused an inhibition of dTMP synthetase in crude extracts from

phage-infected cells could have acted in several different ways. Before concluding that it acted directly on dTMP synthetase, and investigating the interaction in detail it was necessary to exclude the following two possibilities.

1. That the protein was an enzyme which acted on the substrates or products of dTMP synthetase thereby invalidating the assay of dTMP synthetase. For example, Haussman and Gold (1966) found that although infection of E. coli by phage T3 caused an apparent large decrease in the activity of a DNA methylating enzyme, the decrease was caused by the induction of a second enzyme, which invalidated the assay for the DNA methylase by destroying the methyl donor, S-adenosyl-methionine (Gefter, Haussman and Gold, 1966).

The assay for dTMP synthetase depended on the oxidation of H_4PteGlu to H_2PteGlu which could be followed spectrophotometrically. Any enzyme which utilised one or both of the substrates of dTMP synthetase, or which reduced the product H_2PteGlu back to H_4PteGlu could invalidate the assay. The phage-induced dUMP hydroxymethylase would be included in the first category and dihydrofolate reductase in the second.

It was unlikely that the inhibitor affected the substrates of dTMP synthetase, both because these

could be incubated with the inhibitor without affecting their subsequent ability to function in the dTMP synthetase assay, and because fractionation experiments showed that the inhibitor was not identical with the dUMP hydroxymethylase. Dihydrofolate reductase was not responsible for causing an apparent inhibition because purified samples of the inhibitor inactivated purified dTMP synthetase without the necessary addition of any reduced pyridine nucleotides.

These possibilities were completely eliminated by fractionation experiments. Although the inhibitor could be separated from uninhibited dTMP synthetase when an artificial mixture of both was fractionated, only the residual traces of dTMP synthetase could be recovered when the technique was applied to extracts from infected cells. Therefore the inhibitor did not specifically invalidate the assay for dTMP synthetase.

2. That the protein was an enzyme which acted on an unknown substrate to give a product which was the direct inhibitor of dTMP synthetase. This possibility would have explained why a mixture of extracts from infected and uninfected cells required a preincubation period before any inhibition of dTMP synthetase was observed. Several natural inhibitors of dTMP synthetase are known. Blakley (1963) reported that the products of the

reaction, dTMP and H_2PteGlu inhibit dTMP synthetase, and Friedkin et al.(1962) isolated an inhibitor which was described as a folic acid analogue, during the purification of dTMP synthetase from E. coli.

The inhibition of dTMP synthetase in phage-infected extracts was not reversed by dialysis, and was therefore not caused by any reversible inhibitor such as dTMP. This experiment did not exclude an inhibitor of the type described by Friedkin et al.(1962), which was separated from E. coli dTMP synthetase only by DEAE-cellulose chromatography. If a low molecular weight inhibitor of dTMP synthetase were formed in situ during the joint incubation of extracts from infected and uninfected cells, then preincubation of the infected extract alone should also generate the inhibitor. In fact when extracts from infected cells were incubated alone, the inhibitory activity of the extracts was decreased rather than enhanced. Experiments using purified inhibitor and dTMP synthetase showed that the inhibition occurred in the absence of any low molecular weight substance which could be converted to an inhibitor.

It was concluded from these results that the protein inhibitor acted directly on dTMP synthetase.

Section 2. The mechanism of the inhibition process

The inhibitor could inactivate dTMP synthetase either by a catalytic mechanism or by forming a stable complex with the enzyme (stoichiometric mechanism). Since the experimental results did not distinguish unambiguously between the two types of mechanism, the interaction of the inhibitor and dTMP synthetase is compared with a number of possible model systems.

Models for an enzymatic mechanism

A number of different enzyme-catalysed processes could result in the inactivation of dTMP synthetase. The inhibitor might be a proteolytic enzyme. If it were a protease of wide specificity it would be expected to destroy other enzymes in addition to dTMP synthetase. Yet the phage-induced enzymes dCMP deaminase (Roscoe and Tucker, 1966) and the dUMP hydroxymethylase may be assayed normally in extracts containing the inhibitor, and Roscoe (1965) found that dihydrofolate reductase activity was unaltered after phage infection. In addition, preliminary experiments in which the inhibitor was incubated with malic dehydrogenase, glucose-6-phosphate dehydrogenase, or E. coli dTMP synthetase suggested that only the last was affected by the inhibitor. Therefore, if the inhibitor is a protease it appears to be specific for dTMP synthetase. Breakage of a single peptide bond

might be sufficient to inactivate the enzyme. Such a mechanism would be analoguous to the activation of trypsinogen by enterokinase, in which a small hexapeptide is split off by cleavage of the bond between lysine and isoleucine (Dixon and Webb, 1958).

As an alternative to proteolysis the inhibitor might theoretically cause a number of other modifications of the dTMP synthetase molecule by attacking amino acid side chains or the terminal amino acids. Since little is known about the primary, secondary or tertiary structure of dTMP synthetase protein, or about the nature of the catalytic site, such possibilities are entirely speculative.

A mechanism for controlling enzyme activity by a catalytic process which has been thoroughly investigated involves phosphorylation and dephosphorylation. The former reaction is catalysed by an ATP-linked kinase and the latter reaction by a phosphatase. The activities of glycogen synthetase and glycogen phosphorylase are controlled by this mechanism (e.g. Stadtman, 1966). If dTMP synthetase activity in uninfected cells were similarly controlled, the balance between active and inactive forms might well be altered by phage infection. However, the possibility is unlikely because there are no reports that dTMP

synthetase activity is controlled in this way, and there is no evidence that the inhibitor of dTMP synthetase (which would correspond to the phosphatase of the above mechanism since it has no ATP requirement) is present in the uninfected cell.

Models for a stoichiometric mechanism

The formation of complexes between proteins may result in both activation or inactivation of enzymes. For example, fumarase is composed of four similar protein subunits which are enzymically inactive until associated to form a tetramer (Hill and Kaplan, 1964), and conversely a number of protein inhibitors are known to form complexes with the enzymes they inhibit. Perhaps the most familiar and most highly purified protein inhibitors are the various inhibitors of trypsin (e.g. Laskowski and Laskowski, 1954). The first trypsin inhibitor was isolated by Kunitz and Northrop (1936), and the system now serves as a model for studying protein-protein interactions. Trypsin forms a 1:1 complex with its inhibitors and the dissociation constants of the various complexes are of the order of 10^{-10} M (Green, 1953) at the optimum pH for association.

Other presumptive protein inhibitors which have been described include (i) an inhibitor of pancreatic deoxyribonuclease (Lindberg, 1964); (ii) an inhibitor of mammalian ribonuclease (Shortman, 1961,

1962); (iii) an inhibitor of DPN nucleotide pyrosphosphatase isolated from Proteus vulgaris (Swartz, Kaplan and Lamborg, 1958); and (iv) an inhibitor of ornithine transcarbamylase isolated from yeast (Bechet and Wiame, 1965). In addition to these examples, the interaction between an enzyme and its specific antibody may result in the loss of enzymatic activity, and involves the formation of complexes between the proteins concerned.

The interaction of dTMP synthetase with its inhibitor could be similar to any of these examples. Although many protein inhibitors appear to react almost instantaneously with the enzyme they inhibit, protein protein interaction are not necessarily rapid processes, and the measurable rate of the interaction of dTMP synthetase and the inhibitor in vitro does not exclude a stoichiometric type of mechanism. The association of fumarase subunits (Hill and Kaplan, 1964) and the interaction of the basic pancreatic inhibitor with trypsin (Green, 1957) both appear to occur at comparably slow rates, although it is not possible to compare specific rate constants.

Discussion of the experimental results in relation to the above models

A. The biphasic progress curve

1. The interaction of dTMP synthetase with the

inhibitor was investigated at two levels of purity. Most experiments were carried out using ammonium sulphate fractions of the inhibitor and dTMP synthetase. The preliminary results using more highly purified samples of both proteins tended to confirm the results obtained with the less pure system. The reaction was biphasic both at 25° and at 37°, an initial fast reaction being followed by a slower one. Five minute incubations, which were assumed to be an approximate measure of the rate of the fast reaction, showed that the rate was dependent on the concentrations of both proteins, except when either was present in a presumed large excess. Preliminary results also suggested that the rate of the initial process increased with increases in pH over the range pH 6-9. Measurements of the rate of inhibition at pHs beyond pH9 have not yet given reproducible results.

Had the inhibitor in the ammonium sulphate fraction been stable to the conditions of incubation it would have been feasible to follow the interaction to completion, and hence deduce the probable mechanism. Since the inhibitor was unstable the interpretation of the progress curve of the reaction was difficult, though the following possibilities were considered.

1. That the changing reaction rate was due to

the decreasing concentration of unreacted material (stoichiometric model), or to the instability of the catalytic inhibitor (enzyme model). The inhibitor was unstable when it was incubated alone at 37° and the largest decreases in activity occurred during the first 10 min. This would correspond to the abrupt change in reaction rate at 10 min when the inhibitor was incubated with dTMP synthetase, although the possibility that the inhibitor is stabilised in the presence of dTMP synthetase cannot be discounted.

2. That two forms of either the inhibitor or of dTMP synthetase existed. In either case a biphasic reaction rate might be observed whatever the mechanism of the interaction.

There was some evidence that dTMP synthetase was heterogeneous. The fractionation of the enzyme from B. subtilis on DEAE-cellulose indicated that two peaks of activity might exist, and Wahba and Friedkin (1962) also found three quite distinct peaks of E. coli dTMP synthetase activity during a similar fractionation. In both cases this preliminary evidence requires confirmation by rechromatography of the individual peaks and in neither case has such work been done. If two forms of dTMP synthetase were responsible

for the biphasic kinetics then the second phase rates observed in the inactivation of a constant amount of enzyme by different amounts of inhibitor should extrapolate back to the same point on the zero time axis. This would show the presumably constant ratio of the two forms of dTMP synthetase. The available experimental evidence did not confirm this.

The existence of two forms of the inhibitor is possible, although it fractionated as a single peak on DEAE-cellulose.

3. That the interaction of dTMP synthetase and the inhibitor proceeded in two consecutive stages. If the inhibitor acts enzymically the two stages might correspond to the formation of the enzyme substrate complex and to the subsequent enzymic reaction. If the inhibitor acts stoichiometrically two distinct stages of inactivation are involved and dTMP synthetase is only partially inactivated in the first step. A two-stage mechanism for the interaction of the soybean trypsin inhibitor with trypsin has been proposed (Finkenstadt and Laskowski, 1965).

In conclusion, the investigation of the interaction of the inhibitor and dTMP synthetase did not indicate the mechanism of the process. However, since the sucrose-concentrated purified sample of the inhibitor

appeared to be stable to incubation at 37° it may prove useful in similar experiments in the future.

B. The study of the inhibition achieved at different pHs

If dTMP synthetase could be reactivated after inhibition, the mechanism of the inhibition process would be stoichiometric rather than catalytic. When phage-infected cells were sonicated at pH6 70-100% of the dTMP synthetase activity was recovered. Assuming that the enzyme and inhibitor are associated *in vivo* this result implied that the interaction was reversible, and thus favoured the stoichiometric mechanism. The validity of this assumption is discussed later in Section 3.

The same tentative conclusion was suggested by the variation in dTMP synthetase activity found when infected cells were disrupted at a range of different pHs. Three different levels of dTMP synthetase activity were observed in extracts from cells sonicated at pHs 6, 7 and 7.5, despite the presence of excess inhibitor in all cases. The preliminary experiments on the effect of pH on the interaction *in vitro* of dTMP synthetase and the inhibitor showed that the inhibition occurred at pHs 6 and 7 but at a very slow rate. However the residual dTMP synthetase activity in the sonicated extracts did not change over a 24h period, which suggested that the pH of the medium also affected the

equilibrium position of the reaction.

The interaction of the basic pancreatic trypsin inhibitor with trypsin is closely analoguous to the above results. Green and Work (1953) and Green (1957) found that both the rate and the percentage formation of the trypsin-inhibitor complex were similarly affected by pH. Therefore by analogy, the inhibitor of dTMP synthetase probably formed a complex with the enzyme, although this argument does not rigorously exclude the alternative enzymic mechanism.

The proposed complex of inhibitor and dTMP synthetase, once formed, was not easily dissociated to give enzymically active dTMP synthetase, although a dissociation to give inactive dTMP synthetase would be undetectable by the methods used. Conditions which destroyed excess free inhibitor, such as fractionation of infected-cell extracts with solid ammonium sulphate, or incubation of infected-cell extracts overnight at 37°, did not cause any reversal of the inhibition by re-equilibration.

These failures to reactivate dTMP synthetase <u>in vitro</u> do not prove that it is impossible. A number of potential methods have not yet been investigated, and in addition it is possible that dissociated dTMP

synthetase is enzymically inactive and requires a particular environment for reactivation. This might explain why dialysis of inhibited dTMP synthetase to pH6 did not result in activation, yet sonication of infected cells at this pH gave active enzyme.

Proposed methods of confirming the mechanism of the interaction

The mechanism of the interaction of dTMP synthetase and the inhibitor could be further investigated in at least three different ways.

1. Further attempts to reactivate dTMP synthetase

The proposed complex of inhibitor and enzyme might be dissociated by heat treatment. Thus Bechet and Wiame (1963) dissociated a protein inhibitor from ornithine transcarbamylase by incubating the inhibited enzyme at 60° for 5 min. Similarly, Swartz et al. (1958) dissociated an inhibitor from DPN nucleotide pyrophosphatase by incubating the inhibited enzyme at 100° for 2 min.

An alternative to heat treatment would be the use of reagents such as urea, detergents and guanidine hydrochloride which cause dissociation of protein complexes (Tanford, 1964). Since these reagents are likely to inactivate dTMP synthetase, a preliminary investigation of their effect on the enzyme alone would be necessary to check that such inactivation was reversible.

2. A physical study of the interaction

The most satisfactory method of investigating the interaction of the inhibitor with dTMP synthetase would be by physical methods using purified proteins. This was the ultimate intention of the purification work. A 30 fold purification of dTMP synthetase was achieved by conventional methods of enzyme purification. The results of the analytical polyacrylamide gel electrophoresis suggested that a preparative application of this technique to the purified enzyme would result in a considerable further purification. The prospect of obtaining a pure sample of dTMP synthetase is therefore promising.

The purification of the inhibitor was less well advanced and the prospect of obtaining a completely pure sample is more remote. If a complete purification of dTMP synthetase were available it should be possible to isolate a radioactive enzyme from B. subtilis grown on labelled amino acids. The radioactive label would provide a means of identifying inhibited dTMP synthetase and the interaction with the partially purified inhibitor could then be studied by physical methods such as ultracentrifugation, gel filtration and polyacrylamide gel electrophoresis.

3. The exchange reaction catalysed by dTMP synthetase

Protein modifications do not necessarily affect

simultaneously all the enzymic activities of an enzyme which catalyses more than one process. For example, Mecke et al.(1966) described a protein inhibitor of glutamine synthetase which affects only part of the active site of the enzyme leaving glutamyl transferase activity intact. Lomax and Greenberg (1967a and b) have suggested that the reaction catalysed by dTMP synthetase proceeds by the reversible formation of a methylene bridge between dUMP and H_4PteGlu, followed by an irreversible rearrangement to give the products (Fig. 30). The reversible first stage may be distinguished from the overall reaction since in tritiated water it results in an incorporation of label into dUMP. It would be interesting to discover how the inhibitor of dTMP synthetase affected this exchange reaction.

Section 3. The cellular origin and physiological role of the inhibitor of dTMP synthetase

The origin of the inhibitor

The inhibitor of dTMP synthetase behaved like a phage-induced protein. Thus the inhibition was undetectable five minutes after the addition of phage Øe and the amount of inhibitor present increased for at least the first 25 min of the infection. In addition, the results showed that protein synthesis was necessary for the appearance of the inhibition and Roscoe (1965)

Figure 30

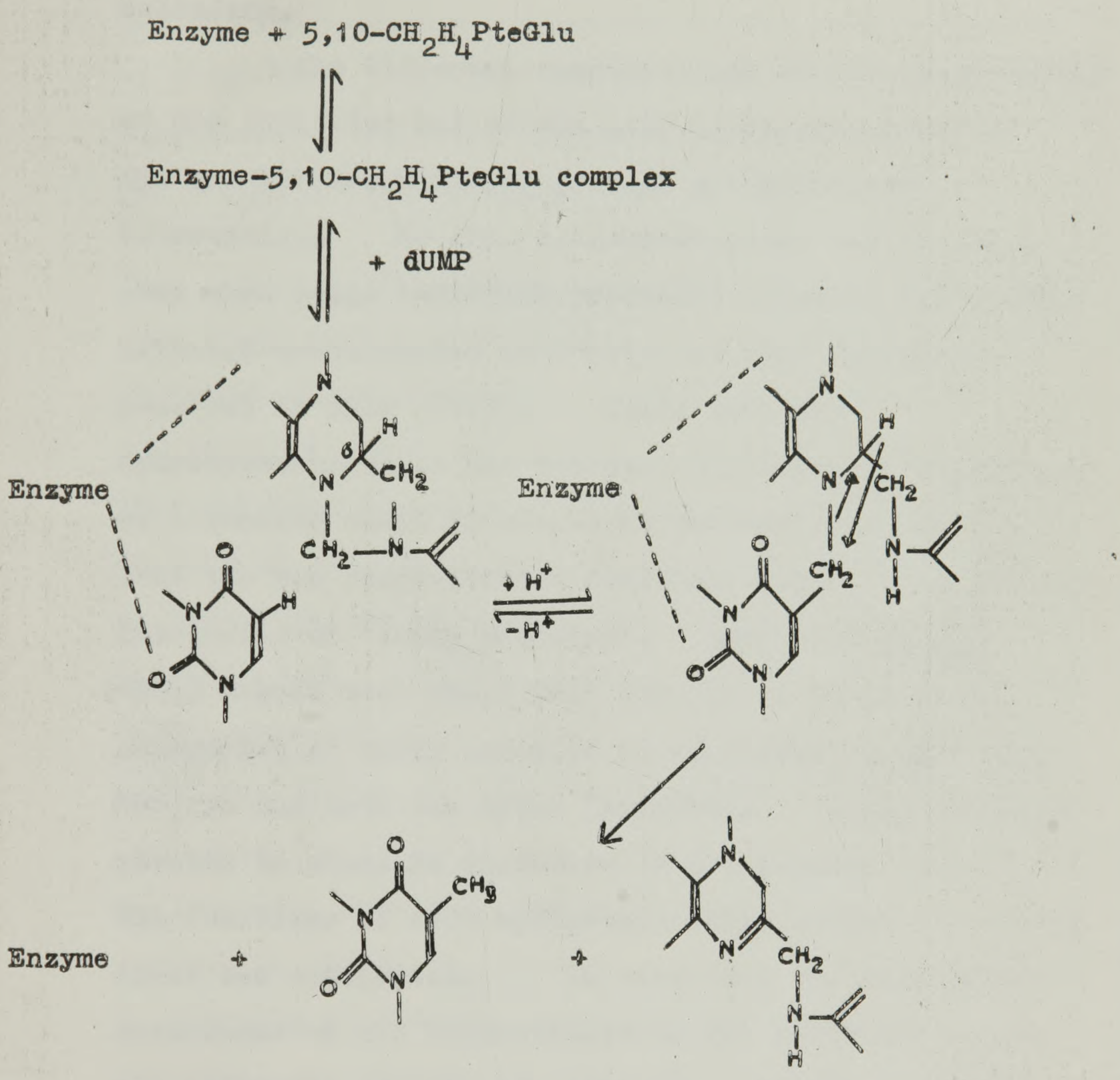


Fig. 30. Proposed mechanism for the action of dTMP synthetase (Lomax and Greenberg, 1967b)

See Fig. 2 for the complete chemical structures.

showed that DNA-dependent RNA synthesis was also necessary.

The different sensitivities of the biosynthesis of the inhibitor and of the dUMP hydroxymethylase to the conditions inhibiting protein synthesis were interesting. No dUMP hydroxymethylase was detected even when phage infection proceeded normally for 5 min, although considerable inhibition of dTMP synthetase occurred in this period. Since the dUMP hydroxymethylase is not detected under normal conditions of infection until 7½ min, it is reasonable to assume that the two phage-induced functions belong to different categories of 'early proteins'. Levinthal et al. (1966) showed that phage SP01 induced at least three categories of early proteins at approximately 2-4 min, 4-6 min and 8-10 min after infection. Applying these results to phage Øe infection it would appear that the inhibitor of dTMP synthetase falls within one of the first two categories. In this case the comparative resistence of the biosynthesis of the inhibitor to the two different methods of inhibiting protein synthesis could be attributed to the preferential synthesis of a very small number of proteins at the start of phage infection.

Although the circumstantial evidence that the inhibitor is a phage-induced protein is strong, this

conclusion cannot be confirmed until a structural gene for the inhibitor is located within the genetic map of phage Øe. In view of the uncertainty about its physiological role two alternative explanations for the origin of the inhibitor must be considered.

1. That the inhibitor is coded for by a bacterial gene which becomes derepressed on phage infection. As discussed in the Introduction some host functions may continue during infection by phages SP01 and SP8. This may also be the case in phage Ø e infection. The ability of the host genome to direct the synthesis of an inducible enzyme during phage infection has been investigated. Synthesis of histidase ceases 5-10 min after phage infection (Roscoe, private communication). Since the amount of inhibitor in phage-infected cells increases for at least the first 25 min of phage infection, the inhibitor does not appear to resemble this particular inducible enzyme. However histidase in B. subtilis is subject to catabolite repression (Hartwell and Magasanik, 1963) and if phage infection altered the metabolism of the host cell such that there was a rise in the pool of intermediates, histidase synthesis might cease, (compare effect of 5-fluorouracil on the induction of β-galactosidase, Horowitz and Kohlmeier, 1967). Further investigation of the effect

of phage infection on the induction of a host enzyme not subject to catabolite repression is therefore desirable.

2. That a preexisting inhibitor is activated or released as a result of phage infection. This possibility is considered because it is a reasonable explanation of the confusing reports on the inhibition of DNA-dependent RNA polymerase during infection of E. coli by phages T2 or T4 (Introduction). There was no evidence for any traces of the inhibitor in uninfected B. subtilis. The possibility could be completely eliminated once a purification of the inhibitor is available, by demonstrating that the inhibitor protein is synthesised de novo after phage infection.

The activity of dTMP synthetase in vivo

The activity of dTMP synthetase in vivo cannot be deduced from the activity observed in cell-free extracts since the latter is dependent on the pH of sonication. This problem is related both to the mechanism of the inhibition process and to the origin of the inhibitor.

If the inhibition process occurs by an irreversible mechanism it does not occur in vivo because infected-cell extracts contained normal amounts of dTMP synthetase when sonicated at pH 6. If the inhibition process occurs by a reversible mechanism there

are two possible alternatives for the activity of dTMP synthetase *in vivo*.

1. Thymidylate synthetase is not inhibited *in vivo* and the inhibitor and dTMP synthetase are brought together by cellular disruption. This explanation was proposed by Swarts *et al.* (1958) to explain their finding that although whole cells of *Proteus vulgaris* are able to cleave DPN at the pyrophosphate bond, in sonicated extracts the enzyme DPN nucleotide pyrophosphatase was inhibited by a protein inhibitor.

2. Thymidylate synthetase is inhibited *in vivo* and the complex with the inhibitor is dissociated by the conditions of cellular disruption. For example, Bechet and Wiame (1965) observed that when the activity of ornithine transcarbamylase was measured in whole 'permeabilised' yeast cells it was inactivated by a protein inhibitor under conditions of arginine repression. However, if the enzyme was assayed in cell-free extracts no inhibition was observed. In the case of dTMP synthetase the final activity of the enzyme in cell-free extracts would be due to a pH-dependent reassociation with the inhibitor.

It is proposed that if the inhibition of dTMP synthetase occurs *in vivo*, the inhibitor may be

a phage-induced protein with a definite physiological role and must interact with dTMP synthetase by a reversible mechanism. However, if the inhibition occurs only in cell-free extracts the probable origin and physiological role of the inhibitor are less obvious. The most direct way to confirm the former proposition would be to devise an assay for dTMP synthetase *in vivo*.

The physiological role of the inhibitor

Assuming that the inhibition occurs *in vivo* what is the importance of the inhibition in phage infection? The inactivation of dTMP synthetase will both prevent the synthesis of dTMP and cause the accumulation of dUMP.

Although thymine nucleotides may accumulate in the cell by a number of pathways, Wilson *et al.* (1966) suggested that dTMP synthetase was the most important. Therefore its inhibition will have a considerable effect on the intracellular concentration of dTMP and hence of dTTP. However the inhibitor is unlikely to be the only mechanism induced by phage Øe to block thymine biosynthesis for two reasons. Firstly because thymidylate may be formed by pathways other than dTMP synthetase (Introduction) and secondly because phage infection of a thymine-requiring host is normal. The exogeneous thymidine supplied to this mutant during the infection would be expected to bypass the lack of

dTMP synthetase and become incorporated into phage DNA. By analogy with phage SP8, which induces a dTTPase (Kahan et al. 1964) and was also observed to cause some inhibition of dTMP synthetase, it is probable that the second block in thymine biosynthesis induced by phage Øe is a dTTPase. This is confirmed by Roscoe (private communication) who finds that a dTTPase is induced 7½ min after the addition of phage Øe, but that there is no evidence for the induction of a dTMPase.

Why do phages Øe and SP8 induce both a dTTPase and cause an inhibition of dTMP synthetase? The importance of the inhibition could lie in the timing. Since the inhibitor is detectable after 5 min of phage infection, dTMP synthetase may be considerably inhibited before the simultaneous appearance at 7½ min of the dTTPase and the dUMP hydroxymethylase. The end product of the action of both dTMP synthetase and the dTTPase is dTMP which would accumulate if dTMP synthetase were not inhibited. Therefore the quicker dTMP synthetase is inhibited the less dTMP will ultimately accumulate. Thymidylate has no known role in the economy of a cell devoted to synthesising DNA containing hydroxymethyluracil and may even be inhibitory. For example, dTMP has been shown to inhibit the phage-induced dCMP deaminase (Roscoe and

Tucker, 1966).

The probable accumulation of the substrates of dTMP synthetase resulting from its inhibition is also advantageous since they are utilised by the vital phage-induced dUMP hydroxymethylase. An active dTMP synthetase would divert some dUMP and H_4PteGlu away from the biosynthesis of hydroxymethyluracil.

The possible physiological roles of the inhibition of dTMP synthetase would probably have only marginal effects on the success or failure of phage Øe infection. However this is also true of at least two of the enzymes induced by phage T4 in E. coli. Mutants of T4 have been obtained which grow well on the normal host yet cannot induce the virus-specific dTMP synthetase (Simon and Tessman, 1963) or the dCMP deaminase (Hall and Tessman, 1966). The burst size of the former mutants is normal but the burst size of the latter type of mutant is lower than that of the wild-type phage. It is probable that such marginal effects give the phage a slight selective advantage.

In this context the following observations may be significant. (i) Phage SP8, which causes a slower and less effective inhibition of dTMP synthetase when compared with phage Øe, can multiply in B. subtilis 168 but not in B. subtilis 3610. Yet phage SP8 causes lysis of the latter strain at low multiplicities of infection.

The specific activity of dTMP synthetase in extracts from B. subtilis 3610 is nearly double the activity in extracts from B. subtilis 168.

(ii) The succesful infection of B. subtilis 3610 by phage Øe in nutrient broth is also finely balanced. Roscoe (1965) found that reproducible multiplication occurred only if the bacteria were infected at the beginning of the logarithmic phase of growth. This corresponds to a comparatively low specific activity of dTMP synthetase.

Since there are numerous alternative explanations for both the above phenomena a thorough investigation is required to consider the possibility that the inhibition of dTMP synthetase may be a common factor.

Summary

The inhibition of dTMP synthetase which follows the infection of B. subtilis by phage Øe is due to the synthesis of a protein inhibitor of dTMP synthetase. The inhibitor acts directly on the enzyme and a dissociable complex of the two proteins may be formed. Since the complex has not yet been dissociated in vitro the possibility that the inhibitor is a specific inactivating enzyme cannot be completely excluded.

The inhibitor may have a role *in vivo* provided the inhibition of dTMP synthetase is effective under physiological conditions, but is reversed by sonic disruption of infected cells. Infection by phages Øe and SP8 causes the induction of a dTTPase in addition to causing an inhibition of dTMP synthetase. Therefore the role of the inhibitor *in vivo* may be a marginal one of reducing the probable accumulation of intracellular thymidylate, and facilitating the efficient utilisation of dUMP by the phage-induced enzyme dUMP hydroxymethylase.

REFERENCES

Adams, M.H. (1959). in Bacteriophages, New York: Interscience.

Ames, B.N. and Martin, R.G. (1964). Ann. Rev. Biochem. 33, 235.

Anagnostopoulos, C. and Schneider-Champagne, A. (1966). C.R. Acad.Sci. Paris, 262, 1311.

Aposhian, H. V. (1965). Biochem. biophys. Res. Commun. 18, 230

August, J.T., Ortiz, P.J. and Hurwitz, J. (1962). J. biol. Chem. 237, 3786.

Bechet, J. and Wiame, J.M. (1965). Biochem. biophys. Res. Commun. 21, 226.

Blakley, R.L. (1963). J. biol. Chem. 238, 2113.

Blakley, R.L. and McDougall, B.M. (1962). J. biol. Chem. 237, 812.

Blakley, R.L., Ramasastri, B.V., and McDougall, B.M. (1963). J. biol. Chem. 238, 3075.

Brabander, W.J. and Romig, W.R. (1960). Bacteriol. Proc. p187.

Bradley, D.E. (1965). Journal of the Royal Microscopical Society, 84, Part 3, 257.

Burton, K. (1956). Biochem. J. 62, 315.

Cairns, J. (1961). J. Mol. Biol. 3, 756.

Clarke, J.T. (1964). Ann. N.Y. Acad. Sci. 121, 428.

Cline, A.L. and Bock, R.M. (1966). Cold Spring Harbor Symp. Quant. Biol. 31, 321.

Davidson, J.N. (1965). in The Biochemistry of the Nucleic Acids, (5th Edition), p169, London: Methuen & Co. Ltd.

Davis, B.J. (1964). Ann. N.Y. Acad. Sci. 121, 404.

Davis, B.D. and Mingioli, E.S. (1950). J. Bact. 60, 17.

Davis, J.E., Strauss, J.H. and Sinsheimer, R.L. (1961). Science, 134, 1427.

Davison, P.F. (1963). Virology, 21, 146.

Dilworth-Cannon, W. and Breitman, T.R. (1967). Biochemistry, 6, 810.

Dixon, M. and Webb, E.C. (1958). in The Enzymes, p546 London: Longmans.

Eiserling, F.A. and Romig, W.R. (1964). Bacteriol. Proc. p118.

Ellis, E.L. and Delbrück, M. (1939). J. gen. Physiol. 22,365.

Farmer, J.L. and Rothman, F. (1965). J. Bact. 89, 262.

Ferguson, J.J., Boll, M. and Holzer, H. (1967). European J. Biochem. 1,21.

Finkenstadt, W.R. and Laskowski, M. (1965). J. biol. Chem. 240, PC962.

Flaks, J.G. and Cohen, S.S. (1959). J. biol. Chem. 234, 1501.

Friedkin, M. , Crawford, E.J., Donovan, E. and Pastore, E.J. (1962). J. biol. Chem. 237, 3811.

Furth, J.J. and Pizer, L.I. (1966). J. Mol. Biol. 15, 124.

Gage, L.P., Wilson, D.L. and Geiduschek, E.P. (1967). Fed. Proc. 26, 873.

Gefter, M., Hausseman, R., Gold, M. and Hurwitz, J. (1966). J. biol. Chem. 241, 1995.

Gomori, G. (1948). Proc. Soc. Exptl. Biol. Med. 68, 354.

Green, D.M. (1964). J. Mol. Biol. 10, 438.

Green, N.M. (1953). J. biol. Chem. 205, 535.

Green, N.M. (1957). Biochem. J. 66,407.

Green, N.M. (and Work, E. (1953). Biochem. J. 54, 347.

Greenberg, D.M., Nath, E. and Humphreys, G.K. (1961). J.biol. Chem. 236, 2271.

Hall, D.H. and Tessman, I. (1966). Virology, 29, 339.

Hall, C.E., Maclean, E.C. and Tessman, I. (1959). J. Mol. Biol. 1, 192.

Hartwell, L.H. and Magasanik, B. (1963). J. Mol. Biol. 7, 401.

Hausesman, R. and Gold, M. (1966). J. biol. Chem. 241, 1985.

Hershey, A.D. and Chase, M. (1953). J. gen. Physiol. 36, 39.

Hill, R.L. and Kanarek, L. (1964). in Subunit Structure of Proteins, Brookhaven Symposia in Biology, 17, 80.

Horowitz, J. and Kohlmeier, V. (1967). Biochim. biophys. Acta, 142, 208.

Ikeda, Y., Saito, M., Miura, K., Takagi, J. and Aoki, H. (1965). J. Gen. Appl. Microbiol. (Tokyo), 11, 181.

Jacob, F. and Monod, J. (1961). J. Mol. Biol. 3, 318.

Jenny, E. and Greenberg, D.M. (1963). J. biol. Chem. 238, 3378.

Jones, K.M., Guest, J.R. and Woods, D.D. (1961). Biochem. J. 79, 566.

Kahan, F., Kahan, E. and Riddle, B. (1964). Fed. Proc. 23, 318.

Kallen, R.G., Simon, M. and Marmur, J. (1962). J. Mol. Biol. 5, 248.

Kay, D. (1962). J. gen. Microbiol. 27, 201.

Kay, D. and Bradley, D.E. (1962). J. gen. Microbiol. 27, 195.

Keck, K., Mahler, H.R. and Fraser, D. (1960). Arch. Biochem. Biophys. 86, 85.

Kellenberger, E. (1961). Adv. Virus Research, 8, 1.

Khesin, R.B., Shemyakin, M.F., Gorlenko, Z.M., Bogdanova, S.L. and Afanas'eva, T.F. (1962). Biokhimiya, 27, 1092.

Kleinschmidt, A.K., Lang, D., Jacherts, D. and Zahn, R.K. (1962). Biochim. biophys. Acta, 61, 857.

Knight, B.C.J.G. and Proom, H. (1950). J. gen. Microbiol. 4, 508.

Kornberg, A., Zimmerman, S.B., Kornberg, S.R. and Josse, J. (1959). Proc. nat. acad. Sci., Wash. 45, 722.

Kunitz, M. and Northrop, J.H. (1936). J. gen. Physiol. 19, 991.

Kuno, S. and Lehman, I.R. (1962). J. biol. Chem. 237, 1266.

Larsson, A. and Reichard, P. (1966). J. biol. Chem. 241, 2533.

Laskowski, M. and Laskowski, M. (1954). Advan. Protein Chem. 9, 203.

Lennox, E.S. (1955). Virology, 1, 190.

Levinthal, C., Fan, D.P., Higa, A. and Zimmerman, R.A. (1963). Cold Spring Harbor Symp. Quant. Biol. 28, 183.

Levinthal, C., Hosoda, J. and Shub, D. (1966). Proceedings of the Symposium on the Molecular Biology of Viruses, Canada.

Levinthal, C., Keynan, A. and Higa, A. (1962). Proc. nat. acad. Sci., Wash. 48, 1631.

Lindberg, U. (1964). Biochim. biophys. Acta, 82, 237.

Loeb, T. and Zinder, N.D. (1961). Proc. nat. acad. Sci., Wash. 47, 282.

Lomax, M.I.S. and Greenberg, G.R. (1967a). J. biol. Chem. 242, 109.

Lomax, M.I.S. and Greenberg, G.R. (1967b). J. biol. Chem. 242, 1302.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. biol. Chem. 193, 265.

Lwoff, A. (1957). J. gen. Microbiol. 17, 239.

Maley, G.F. and Maley, F. (1964). J. biol. Chem. 239, 1168.

Mandel, J.D. and Hershey, A.D. (1960). Analyt. Biochem. 1, 66.

Mandelstam, J. (1954). J. gen. Microbiol. 11, 426.

Markham, R. and Smith, J.D. (1949). Biochem. J. 45, 294.

Marmur, J. (1961). J. Mol. biol. 3, 208.

Marmur, J. and Cordes, S. (1963). in Informational Macromolecules, p79. Ed. by Vogel, H.J., Bryson, V. and Lampen, J.O., New York: Academic Press Inc.

Marmur, J. and Greenspan, C.M. (1963). Science, 142, 387.

Marmur, J., Greenspan, C.M., Palacek, E., Kahan, F.M., Levine, J. and Mandel, M. (1963). Cold Spring Harbor Symp. Quant. Biol. 28, 191.

Mecke, D., Wulff, K., Liess, K. and Holzer, H. (1966). Biochem. biophys. Res. Commun. 24, 452.

Mitchell, P. (1949). Nature, Lond. 164, 846.

Monod, J., Wyman, J. and Changeux, J. (1965). J. Mol. Biol. 12, 88.

Okazaki, T. and Kornberg, A. (1964). J. biol. Chem. 239, 259.

Okazaki, R. and Kornberg, A. (1964a). J. biol. Chem. 239, 269.

Okazaki, R. and Kornberg, A. (1964b). J. biol. Chem. 239, 275.

Okubo, S., Strauss, B. and Stodolsky, M. (1964). Virology, 24, 552.

Oleson, A.E. (1966). Fed. Proc. 25, 275.

Ortiz, P.J., August, J.T., Watanabe, M., Kaye, A.M. and Hurwitz, J. (1965). J. biol. Chem. 240, 423.

Pastore, E.J. and Friedkin, M. (1962). J. biol. Chem. 237, 3802.

Pene, J.J. and Marmur, J. (1964). Fed.Proc. 23, 318.

Peterson, E.A. and Sober, H.A. (1962). in Methods in Enzymology, Vol. 5. p3. Ed. by Colowick, S.P. and Kaplan, N.O., New York and London: Academic Press.

Pontis, H .G. and Blumson, N.L. (1958). Biochim biophys. Acta, 27, 618.

Robertson, J.J. and Halvorsen, H.O. (1957). J. Bact. 73, 186.

Romig, W.R. (1962). Virology, 16, 452.

Romig, W.R. and Brodetsky, A.M. (1961). J. Bact. 82, 135.

Roscoe, D.H. (1962). Thesis, Part II, Final Honour School of Chemistry, Oxford.

Roscoe, D.H. (1965). D. Phil. Thesis, Oxford.

Roscoe, D.H. and Tucker, R.G. (1964). Biochem. biophys. Res Commun. 16, 106.

Roscoe, D.H. and Tucker. R.G. (1966). Virology, 29, 157.

Salivar W.O., Tzagoloff, H. and Pratt, D. (1964). Virology, 24, 359.

Scarano, E., Bonaduce, L. and De Petrocellis, B. (1962). J. biol Chem. 237, 3742.

Seaman, E., Tarmy, E. and Marmur, J. (1964). Biochemistry, 3, 607.

Shortman, K. (1961). Biochim. biophys. Acta, 51, 37.

Shortman, K. (1962). Biochim. biophys. Acta, 55, 88.

Simon, E.H. and Tessman, I. (1963). Proc. nat. acad. Sci., Wash. 50, 526.

Sinsheimer, R.L. (1959). J. Mol. Biol. 1, 43.

Sinsheimer, R.L. (1960). in The Nucleic Acids, Vol. III, p187. Ed. Chargaff, E. and Davidson, J.N., New York and London: Academic Press Inc.

Sköld, O. and Buchanan, J.M. (1964). Proc. nat. acad. Sci., Wash. 51, 553.

Spizizen, J. (1958). Proc. nat. acad. Sci., Wash. 44, 1072.

Stadtman, E.R. (1966). Adv. in Enzymol. 28, 41.

Stent, G.S. (1963). in The Molecular Biology of Bacterial Viruses. San Francisco and London: W.H. Freeman and Co.

Stickler, D.J., Tucker, R.G. and Kay, D. (1965). Virology, 26, 142.

Subbaiah, T.V., Goldthwaite, C.D. and Marmur, J. (1965). in Evolving Genes and Proteins, p435. Ed. by Bryson, V. and Vogel, H.J., New York and London: Academic Press Inc.

Swartz, M.N., Kaplan, N.O. and Lamborg, M.R. (1958). J. biol. Chem. 232, 1051.

Takahashi, I. and Marmur, J. (1963). Nature, Lond. 197, 794.

Tanford, C. (1964). in Subunit Structure of Proteins, Brookhaven Symposia in Biology, 17, 154.

Tucker, R.G. (1964). Biochem. J. 92, 58P.

Wahba, A.J. and Friedkin, M. (1961). J. biol. Chem. 236, PC11.

Wahba, A.J. and Friedkin, M. (1962). J. biol. Chem. 237, 3794.

Wilson, M.B., Farmer, J.L. and Rothman, F. (1966). J. Bact. 92, 186.

Witt, I., Kronau, R. and Holzer, H. (1966). Biochim. biophys. Acta, 118, 522.

Wyatt, G.R. and Cohen, S.S. (1953). Biochem. J. 55, 774.