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POLYNUCLEOTIDE PHOSPHORYLASES FROM THERMOPHILES

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

by John N. C. Wood

076.1077

May, 1976

DEDICATED TO MY FAMILY

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## ABSTRACT

A purification procedure was developed for Eschericia coli polynucleotide phosphorylase, and subsequently applied to polynucleotide phosphorylases from Thermus aquaticus and Bacillus stearothermophilus. Preliminary investigations of the catalytic properties of the thermostable polynucleotide phosphorylases were carried out in the hope of effecting the facile polymerisation of modified nucleotide diphosphates which have a predominantly syn conformation. However, even at elevated temperatures, where the relative proportion of substrate molecules in the anti-conformation may be increased, the specificity of the thermostable enzymes was no broader than that reported for mesophylic enzymes. Other catalytic properties investigated were also similar to those observed using polynucleotide phosphorylases from other sources.

Structural studies of the enzyme from B. stearothermophilus revealed a similar gross amino acid composition and molecular weight to the E. coli enzyme. The quaternary structure differs from other polynucleotide phosphorylases in that four apparently identical subunits were identified on polyacrylamide gel electrophoresis under denaturing conditions. The subunits have a molecular weight of 51,000 daltons. Suberimidate cross-linking experiments confirmed a tetrameric structure for the native enzyme. Partially purified polynucleotide phosphorylase from T. aquaticus had a molecular weight of more than 400,000 daltons as judged by gel filtration.

Using a 3' exonuclease from Krebs ascites cells to degrade the rapidly labelled giant nuclear RNA from SV 40 transformed mouse cells, the location of virus specific sequences was investigated by hybridisation to purified SV 40 DNA. An apparent enrichment of virus sequences with increasing degradation of the RNA molecules suggests that virus sequences are absent at the 3' end of giant nuclear RNA.

### ABBREVIATIONS

AMP	Adenosine 5' monophosphate.
ADP	Adenosine 5' diphosphate.
ATP	Adenosine 5' triphosphate.
dADP	Deoxy-adenosine 5' diphosphate.
8brADP	8-bromo adenosine 5' diphosphate.
A <sup>S</sup> DP	8, 2' anhydro-8-mercapto-9-B-D-orabino furanosyladenine-5' -diphosphate.
ATPase	Adenosine-5' -triphosphate phosphohydrolase.
Butyl PBD	2-(4' -tert-butylphenyl )-5-4''-biphenyl)- 1, 3, 4 oxadiazole.
CDP	Cytidine-5' -diphosphate.
Cpm	Comts per minute.
(5i)CDP	5-iodo cytidine-5' -diphosphate.
(5oh)CDP	5-hydroxy-cytidine-5' -diphosphate.
DEAE	Diethylaminoethyl.
DCCD	N, N' -dicyclohexylcarbodiimide.
DMSO	Dimethyl sulfoxide.
DNA	Deoxyribonucleic acid.
DTT	Dithiothreitol.
EDTA	Ethylene diamine tetra-acetic acid.
EcoRI	<u>E. coli</u> restriction endonuclease RI.
GDP	Guanosine 5' diphosphate.
8brGDP	8-bromo-guanosine-5' -diphosphate.
GMP	Guanosine-5' -monophosphate.
HAP	Hydroxylapatite.
HEPES	N-2-hydroxyethylpiperazine-N' -2-ethane- sulfonic acid.

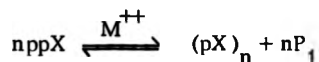
IgG	Immunoglobulin.
(rI)	Ribo-Inosinic acid.
Ka	Half-activation constant.
Ki	Inhibition constant.
Km	Michaelis constant.
MEM	Minimal Eagle's medium.
NAD(H)nm	Nicotinamide adenine dinucleotide nanometry.
OS	Oligomycin-sensitive.
POPOP	2, 2-p-phenylbis(5 phenyloxazole).
PPO	2, 5-diphenyl oxazole.
PMR	Proton magnetic resonance.
HnRNA	Heterogenous nuclear ribonucleic acid.
mRNA	Messenger ribonucleic acid.
pre-mRNA	Precursor messenger ribonucleic acid.
tRNA	Transfer ribonucleic acid.
SDS	Sodium dodecyl sulphate.
SSC	Sodium citrate (0.015 M) sodium chloride (0.15 M) pH 7.0
S	Svedburg unit x $10^{-13}$ s.
SV 40	Simian virus 40.
TCA	Trichloroacetic acid.
TEAE	Triethylaminoethyl.
Tris	Tris(hydroxymethyl)aminomethane.
Oligo(dT)	Oligodeoxythymidilic acid.
UV	Ultraviolet.
UMP	Uridine-5'-monophosphate.
UDP	Uridine-5'-diphosphate.
$5nh_2$ UDP	5-amino uridine-5'-diphosphate.

CHAPTER ONE

1) Discovery and in vitro uses of polynucleotide phosphorylase.

In 1955 polynucleotide phosphorylase (polyribonucleotide: orthophosphate nucleotidyl transferase E.C. 2.7.7.8.) was first detected in Azotobacter agilis by Grunberg-Manago and Ochoa.<sup>1</sup> Since then, a large number of reviews have appeared covering the extensive literature on the enzyme<sup>2-9</sup>, of which the most recent is by Godefroy-Colburn and Grunberg-Manago.<sup>10</sup>

The enzyme, designated PNPase, catalyses the polymerisation of ribonucleotide diphosphates with release of inorganic phosphate. The reaction is fully reversible and can be represented thus:



where ppX is a ribonucleotide diphosphate and  $M^{2+}$  is a divalent metal.

The exchange of the  $\beta$  phosphate of ribonucleotide diphosphates with inorganic phosphate is also catalysed. Catalytic activity is dependent on the presence of a divalent cation, of which magnesium or manganese are the best. Polymerisation may require an RNA primer with a free 3' OH function, or may occur by the de novo polymerisation of two nucleotide diphosphates. Maximum phosphorylase activity occurs with a polynucleotide three or more residues long, with a free 3' OH group.

From the time of its discovery, interest has centred on the use of the enzyme for the polymerisation of nucleotide diphosphates in vitro. The physiological role, catalytic mechanism and structure of the enzyme, have been investigated more recently, and these studies are by no means complete. The experimental uses to which PNPase synthesised polynucleotides have been put are numerous. Examples

include the verification of the Watson-Crick theory of base pairing,<sup>11</sup> the breaking of the genetic code,<sup>12</sup> and the elucidation of many aspects of protein synthesis.<sup>13,14</sup> Mechanistic studies of enzymes concerned with RNA metabolism<sup>15</sup> and structure function studies of RNA inducers of interferon synthesis have also relied on polymers of natural or modified nucleotides,<sup>16</sup> produced by PNPase.

The enzyme also functions as a useful probe for nucleic acid structure, by virtue of the catalytic mechanism of phosphorolysis. Degradation proceeds processively, a single RNA molecule being phosphorolysed without release from the enzyme between successive phosphorolysis steps, whilst the rate of degradation of unstructured RNA is very much faster than that of molecules with ordered secondary or tertiary structure. Information on both the secondary structure of tRNA,<sup>17</sup> and some oligonucleotide sequence data<sup>18</sup> have been obtained, using PNPase.

More recently, the role of poly A tracts in RNA has been investigated by their selective phosphorolysis in globin mRNA<sup>19</sup>, followed by messenger translation in an oöcyte system. The stability of the messenger is significantly lowered after removal of poly A tracts, whilst the addition of poly A tracts leads to a longer half life in the oöcyte.<sup>19</sup>

A number of groups are exploiting PNPase to produce both RNA and DNA of defined sequence. A recent major advance in this area is the development of a biological system for the removal of inorganic phosphate from the polymerisation mixture, developed in Gilham's laboratory.<sup>25</sup> This inhibits PNPase catalysed transnucleotidation which usually results in the desired product being partially converted to smaller and longer chain lengths, with consequent problems in the isolation of the product. The inhibition of transnucleotidation by



phosphate removal confirms the view that the reaction is a combination of phosphorolytic and addition reactions. To produce defined sequence oligo-deoxynucleotides, Gillam and Smith<sup>20</sup> have used PNPase from E. coli which will add a single deoxynucleotide residue to a DNA primer, although some other products are observed. Ribopolynucleotides of defined sequence are produced using 2' or 3' blocked substrates which are re-isolated after addition to a primer, the blocking groups removed and a further round of addition with blocked substrate catalysed.<sup>21</sup>

## 2) Physiological role of PNPase.

All bacteria screened contain PNPase activity.<sup>10</sup> Whether the enzyme is present in all eukaryotes is unknown. Reports of PNPase activity in a variety of plants and animals, including humans, have been made, although low levels of activity are present compared to those observed in prokaryotes. In addition, not all PNPase activities are present in these organisms - for instance, wheat seedlings and human sperm have no phosphorolysis activity, whilst rat and guinea-pig liver will not polymerise nucleotide diphosphates. The localisation of the enzyme in the chloroplasts of bean and cabbage shoots, and the mitochondria of a rat liver has led to the suggestion that the enzyme is associated with systems functioning with 70s ribosomes.<sup>22</sup> However, it should be noted that PNPase activity has been detected both in the nucleolus and nuclear membrane,<sup>23,24</sup> and that the relationship between the ribosomes of prokaryotes and those of mitochondria and chloroplasts is not necessarily a close one.

Bacterial PNPases are usually located in the soluble cytoplasm, although weak binding to ribosomes, probably via RNA and magnesium ions has been observed.<sup>26</sup> In Streptococcus faecalis and Halobacterium cutirubrum, the enzyme is membrane bound.<sup>10</sup>

The metabolic importance of PNPase is emphasised both by its

ubiquity, at least in prokaryotes, and the absence of variable bacterial mutants which contain no PNPase activity. Apirion et al have adopted a genetic approach to try and elucidate the role of the enzyme in vivo, using mutants containing low level or temperature sensitive PNPase activities. Initial studies were carried out on E. coli strains PR7, which contains a thermolabile PNPase, and PR100, its isogenic parental strain.<sup>27</sup> These mutants were isolated by Reiner,<sup>28,29</sup> who devised a screening procedure, utilising the exchange activity of the enzyme to assay heavily mutagenised E. coli strains. A correlation between slowed growth rate and the level of PNPase was observed at 45°C, whilst revertants were found to grow at a similar rate to the parental strain.<sup>29</sup> A decreased ability to induce tryptophanase was also observed at 45°C whilst revertants showed normal behaviour.

A later study was made of both bulk RNA and specific mRNA<sup>30</sup> stability in the two strains, in an effort to localise the lesion in strain PR7. The PNPase from PR7 is claimed to be thermolabile at 48.5°C, and the experiments were carried out at 49°C, at which temperature enzyme induction is still possible. The data presented are claimed to show a destabilisation of mRNA in PR7 at the non-permissive temperature. However, a high proportion of bulk RNA as quantitated by TCA precipitation was stabilised in strain PR7 at 49°C, suggesting a lowered level of RNA turnover. This could explain the lowered level of maximum induction of the enzyme activities investigated, which in turn leads to an apparent increase in the rate of mRNA degradation, when data are analysed by the method of Kepes.<sup>31</sup> Thus a role for PNPase in RNA metabolism is the only firm conclusion that can be drawn from this work. Nevertheless, the authors speculate that PNPase may be involved in mRNA stability, either by effecting the processing of tRNA and rRNA, or by the direct addition of polynucleotides to the 3' end of the mRNA which might cause an

increased resistance to other 3' exonucleases such as ribonuclease II.

The processing of tRNAs does involve 3' exonucleases, as demonstrated by Seidman et al.<sup>32</sup>

In conditions of carbon starvation, PNPase has been shown to catalyse the breakdown of RNA which has been tritium labelled for a long period. This study,<sup>33</sup> also carried out by Apirion, utilised a number of RNase mutants of E. coli which were unfortunately not isogenic. Cells were grown exponentially in a complete medium containing [<sup>3</sup>H] uracil, then transferred to a minimal salts medium, where degradation of RNA was assessed by measurements of TCA soluble counts produced with time. Studies were carried out at various temperatures with strain PR7, which at the non-permissive temperature showed a considerably slower rate of RNA degradation than control strains. Other RNase and PNPase mutants, when analysed in a similar manner, demonstrated the role of PNPase, RNase I and RNase II in RNA breakdown.

Arguments about the role of PNPase have concentrated on the fact that high intracellular levels of inorganic phosphate make a polymerase role for the enzyme unlikely, whilst RNA is degraded from the 5' end, in the opposite direction to that catalysed by PNPase. As no 5' exonuclease has been reported in E. coli, the current view is that mRNA is attacked by endonucleases, which liberate fragments of RNA which exonucleases can then degrade from the 3' end. Both RNase II and PNPase are plausible candidates for this degradative role.

Studies of the showdomycin resistant E. coli strain Show M 500 by Beljanski<sup>34</sup> et al.<sup>35</sup> have led to the hypothesis that PNPase, altered in these strains, plays a role in mRNA synthesis. The viability of these mutants which contain altered ribosomal RNA and protein subunits, as well as low levels of RNA polymerase, is perplexing. Later studies by the same group have suggested that PNPase may be

involved in the synthesis of RNA primers, which greatly enhance the activity of DNA polymerase.<sup>36</sup> These primers, which are purine rich, are only synthesised at 70°C in vitro by wild type PNPase, which makes inactive primers at 37°C. The significance of these observations in ascribing a physiological role to PNPase is questionable.

Biochemical approaches to the role of PNPase in vivo have been made by investigations of the pathway of RNA breakdown in various bacteria by Chaney and Boyer.<sup>37,38</sup> Interestingly, their observations suggest that different pathways of RNA catabolism operate in different bacteria. Thus by [<sup>18</sup>O] RNA labelling studies, they have shown a predominantly hydrolytic breakdown pathway in E. coli whereas Bacillus subtilis shows mainly phosphorolytic degradation. The relative proportions of unstable RNA species in these two strains has been quantitated by Salser, who found a considerable difference between the two.<sup>39</sup>

When grown on identical media, B. subtilis showed a high proportion of unstable RNA, containing 9% of the guanine residues present in the cell, whereas E. coli unstable RNA contained only 3% of the cellular guanine content.

The significance of using well defined growth conditions in any comparative study of microbial metabolism has been emphasised by recent data concerning the presence of poly A residues in prokaryotic mRNA. The original observation of poly A residues attached to rapidly labelled RNA was made by Edwards and Kopp<sup>40</sup>, but was subsequently disputed by Perry et al.<sup>41</sup> The growth conditions of the E. coli strains used differed considerably in their phosphate levels however. While Edwards used medium containing about 10<sup>-1</sup> mM phosphate,<sup>42</sup> Perry used 3x10<sup>-3</sup> M medium containing millimolar phosphate concentrations. Later studies by Srinivasan<sup>44</sup> et al. have showed that up to 15% of rapidly labelled RNA comprises poly A tracts when E. coli is grown on phosphate limiting medium, whilst only 1.5% poly A is found when growth is carried out at high phosphate concentrations. When phosphate limited strains are treated with

higher phosphate concentrations, the addition of further poly A tract<sup>e</sup> to RNA is abolished, although remaining poly A sequences do not appear to be selectively degraded. These studies were carried out on E. coli PR7, which contains no RNase 1, and low levels of PNPase activity. The same group have identified a novel poly A synthesising enzyme which will polymerise either ATP or ADP, is activated by poly A, and at high concentrations of phosphate will phosphorylate poly A<sup>45</sup> to ADP. This activity shows remarkable similarity to one described by Wunderli et al<sup>46</sup> isolated from E. coli K12, and shown to comprise an ATPase and polynucleotide phosphorylase type activity.

Hence, under conditions of phosphate limitation, it is possible that PNPase plays an anabolic role, although there are other enzymes<sup>46</sup> which are equally likely to be responsible for the poly A synthesis observed. The profound effect on RNA anabolism caused by variation of the external levels of phosphate suggest that catabolic pathways might also be effected. It is interesting to note that Chaney and Boyer observed phosphorolytic cleavage in B. subtilis grown on 2 mM inorganic phosphate, whilst E. coli, grown on a minimal salts medium degraded its RNA in a predominantly hydrolytic fashion. The differences between these two observations might be caused by the differing levels of intracellular phosphate resulting from the growth conditions.

Phosphate uptake has been investigated by Medveczky and Rosenberg<sup>47</sup> who have shown the presence of two phosphate uptake systems, a low and a high affinity, which can accumulate phosphate against a concentration gradient, in an energy dependent process. A phosphate pool through which all phosphate taken up passes, is depleted in conditions of phosphate limitation.

It is, therefore, tempting to speculate that the role of PNPase in vivo is directly related to external media conditions. In low phosphate medium, the enzyme restores intracellular phosphate levels by its polymerase action. In conditions of exponential growth, when PNPase is found at twice its usual level in the cell,<sup>4</sup> its role may be to maintain the energy charge of the cell at a suitable level to maintain the rate of various synthetic pathways, by polymerisation of diphosphates, perhaps produced by an associated ATPase activity. In turn, the poly A added to mRNA primers produced, may perhaps by analogy with eukaryotic systems help to stabilise mRNAs under conditions where only a maximal expression of determined gene functions rather than metabolic adaption to the environment is necessary.

This hypothesis suggests an evolutionary origin for mRNA poly A tracts in those organisms which do not require quick metabolic adaption; in other words, multicellular higher eukaryotes.

Under poor growth conditions, the enzyme would predominantly manifest its phosphorolytic activity in response to low levels of nucleotide diphosphates and inorganic phosphate. Hence, no single role could be ascribed to the enzyme without defining the metabolic state of the cell, in relation to its environment. A hopeful development for a more precise investigation of the role of the PNPase is the use of permeabilized cells which can incorporate labelled nucleotide di- and tri-phosphates from the medium.<sup>48</sup>

## (2) Catalytic and mechanistic aspects of the enzyme.

Most of the information concerning PNPases has been obtained from studies of the Micrococcus luteus and E. coli enzymes, and the conclusions drawn seem to be generally applicable.

The enzyme functions between pH 7 and 10.5, the optimum for polymerisation being about pH 9 and for phosphorolysis pH 8. The pH also effects primer dependency, the lower the pH the more dependency is found. Temperature significantly effects not only the rate of the reaction but also the position of equilibrium - for instance, about 60% of ADP is converted to polymer under optimal conditions at 37°C with E. coli PNPase, whilst at 15 - 20°C, up to 80% polymer is produced.<sup>10</sup> Within broad limits ionic strength has little effect on the enzym. e, although Clostridium perfringens PNPase, even in pure preparations, is stimulated by increasing salt concentrations.<sup>49</sup> Other PNPase species sometimes show such an effect in crude preparations, but with purification lose this behaviour. PNPase, isolated from the halophile H. cutirubrum, functions in concentrations of 2 to 4 M salt.<sup>50</sup>

The best metal cofactors are magnesium or manganese II which function with almost equal efficiency, although cobalt, nickel and copper function to a limited extent. Calcium is claimed to be inhibitory.<sup>10</sup> The substrate specificity is somewhat broadened by the use of manganese as compared with magnesium, which allows reactions with presumably unnatural substrates such as deoxynucleotide and various modified ribonucleotide diphosphates to be catalysed.

The specificity of the enzyme is broad. All natural ribonucleotide diphosphates can be polymerised, as can many modified substrates. Even dihydro UDP, which lacks the pyrimidine nucleus, has been polymerised.<sup>10</sup> Polymerisation rate seems to depend to a large extent on the ability of the substrate to attain a suitable orientation around the sugar base bond.<sup>51</sup> Those substrates which are incapable of attaining an anticorformation are found to be either poor substrates or inhibitors. Bulky substituents in the 8 position of purines or the 6 position of pyrimidines tend to promote a syn conformation for steric reasons. (Figure 1). Examples include 8 brGDP and 8 brADP

both of which are very poor substrates for PNPase. Their tight binding as compared with anti-conformation nucleotides has led to the suggestion by Michelson<sup>52</sup> that the catalytic mechanism of the enzyme involves a conformational change of the substrate from anti to syn at the active site. Interestingly, GDP is also a fairly poor substrate, high temperatures and manganese as a cofactor being necessary for satisfactory polymerisation.<sup>53</sup>

Poor substrates can frequently be successfully copolymerised with other ribonucleotide diphosphates, however, by using high concentrations of enzyme and manganese as a cofactor. In some cases, stoichiometric amounts of enzyme to substrate have to be used. This dependence on enzyme concentration may be rationalised by assuming that the oligomer bound to the enzyme may not be capable of assuming a satisfactory conformation for further elongation at the active site of the enzyme to which it is bound, but may be available for a fresh enzyme active site.

The specificity of the enzyme for the ribose moiety is higher than for the base. Phosphorolysis of DNA and polymerisation of deoxynucleotide diphosphates occurs slowly and to a very limited extent.<sup>10</sup> Nevertheless, copolymerisation of dADP and ADP have been reported<sup>54</sup>, whilst only one or two deoxy residues can be added to a primer with no ribonucleotide diphosphates present. Some 2' ribose substituted substrates have been polymerised successfully alone, notably 2'-methoxy ADP<sup>55</sup> and CDP<sup>56</sup>, and also 2'-chloro UDP.<sup>57</sup>

Uridine 5'0(1 thio diphosphate) (Figure 2) has been polymerised by Eckstein,<sup>58</sup> demonstrating that the nature of the polynucleotide phosphate linkage is not crucial to enzymic activity. 2'5' linked UpA has been found to retain priming ability for *M. luteus* PNPase.<sup>59</sup>

As regards the phosphate binding site, it has been shown that arsenate<sup>60</sup> will act as a satisfactory substrate in the phosphorolysis reaction to release readily hydrolysed 5' phosphoryl arsenate nucleosides.

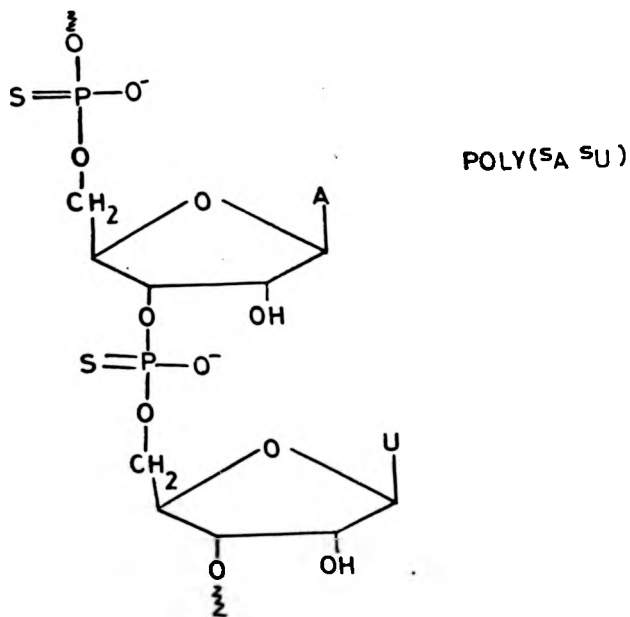
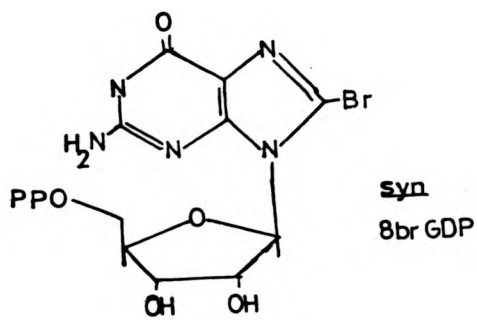
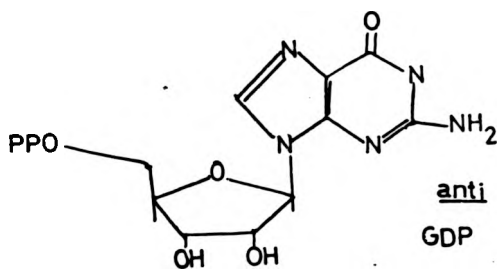


FIGURE 1

Conformation of syn and anti nucleotide diphosphates.

FIGURE 2

Structure of thio substituted poly rA rU



### The phosphorolytic reaction.

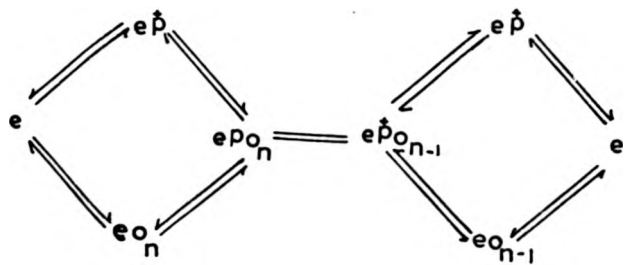
PNPase will phosphorolyse RNAs with free 3' hydroxyl groups. Unstructured RNAs are attacked the fastest, whilst polymers with 3' terminal phosphates are competitive inhibitors. The kinetics of the reaction are dependent on chain length, and are most easily investigated with oligonucleotide substrates, where the reaction proceeds according to Michaelis-Menten kinetics by a rapid equilibrium Bi Bi reaction,<sup>61</sup> with oligonucleotides of various intermediate chain lengths being released during the reaction, (Figure 3). Inorganic phosphate and the oligonucleotide substrate bind independently to the enzyme, the  $K_m$  for phosphate being a constant 0.67 mM for *E. Coli* PNPase,<sup>62</sup> whilst the  $K_m$  values for oligonucleotides vary with chain length. Various kinetic parameters are presented in Table I where it can be seen that optimum binding occurs with an oligonucleotide of about 6 residues long. 2'- and 3'-phosphorylated oligonucleotides equally inhibit the phosphorolysis of oligonucleotides of various chain lengths, and 2 3' cyclic phosphates exhibit the same  $K_i$  as their non-cyclic counterparts, despite the difference in charge.

To explain the dependency of substrate binding on chain length, a model of the active site, with five nucleotide binding sub-sites has been proposed.<sup>65</sup> Evidence for the model is included in Figure 4. Thermodynamic considerations suggest that the sites must be induced on the surface of the enzyme by the substrate, as the difference in binding energies for various sized oligomers is quite small. The data may equally well be explained by the existence of only two sub-sites situated some distance away from each other.<sup>66</sup>

The degradation of RNA molecules longer than ten to twenty residues proceeds in a different fashion to that of oligonucleotides in that a single molecule is degraded processively, without the release of intermediate sized products in the course of the reaction.

FIGURE 3

Reaction mechanism of PNPase.



$e$  = enzyme

$o_n$  = oligonucleotide

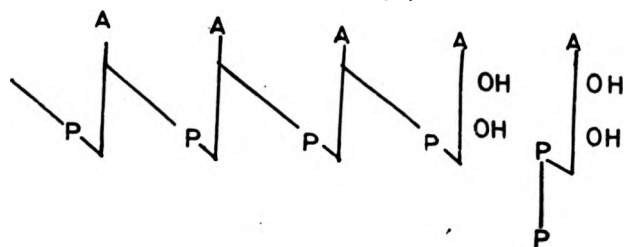
$p$  = phosphate

$p$  = nucleotide  
diphosphate

FIGURE 4

Active site model of PNPase.

POLYMERISATION



PHOSPHOROLYSIS

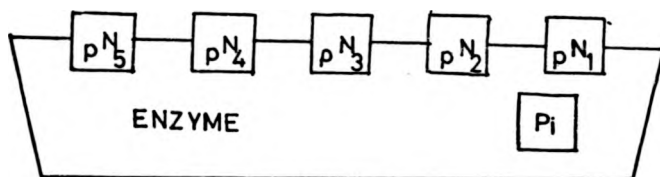
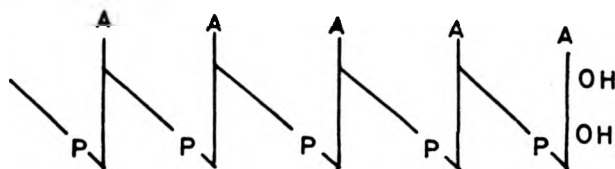


table 1 shows the chain length dependence of binding.

TABLE 1

Kinetic Parameters of Phosphorolysis by PNases<sup>10</sup>

Organism	Substrate	Chain length	Km	V <sub>max</sub> %
<u>E. coli</u>	(pA) <sub>n</sub>	1	-	-
		2	-	-
		3	0.21	100
		4	0.09	100
		5	0.07	100
		6	0.05	56
<u>M. luteus</u>	(Ap) <sub>n-1</sub> A	1	-	-
		2	-	-
		3	2.5	17
		4	0.13	100
		5	0.067	120
		6	0.033	80
		7	0.033	80



This mechanism has also been observed in the exonuclease III isolated from Ehrlich ascites carcinomas.<sup>67</sup> Proof of such a mechanism has been found with PNPase, by using 3' or 5' labelled polymers, where release of TCA-soluble material is consistent with such a mechanism.<sup>68</sup> With tRNA molecules, after partial phosphorolysis, a whole group of undegraded molecules can be isolated.<sup>69</sup> A kinetic model of exonuclease III has been developed by Lazarus et al<sup>70</sup>, which assumes a large number of specific sub-sites, consistent with the large increase in affinity of the enzyme for RNAs of increasing chain length. Such an effect is not pronounced in PNPase however. Oligonucleotide hydrolysis is also processive with the exonuclease, and the kinetics of inhibition of hydrolysis by inhibitor oligonucleotides obeys Michaelis-Menten kinetics, in contrast to PNPase. The exonuclease kinetic model does not therefore seem applicable to PNPase.

With increasing chain length, up to about 40 residues, PNPase binds RNA molecules more tightly than oligonucleotides.<sup>71</sup> Inhibition by 3' phosphate esterified oligonucleotides cannot be completely overcome by the addition of long polymers, and conversely the phosphorolysis of pA 3 is not totally inhibited by the potent inhibitor 3' phosphate poly A.<sup>72</sup> Studies on the time dependence of inhibition of long poly A phosphorolysis by long poly A 3' phosphate show a considerable lag phase, which is considered to mean that the dissociation of the polymer from the enzyme is extremely slow.<sup>73</sup> A satisfactory explanation of these facts has been proposed by Godefroy, who assumes PNPase to have one catalytic site similar to that described in Figure 4, where 3' hydroxy groups and inorganic phosphate bind.<sup>74</sup> A considerable

FIGURE 5

Two site model of PNPase.

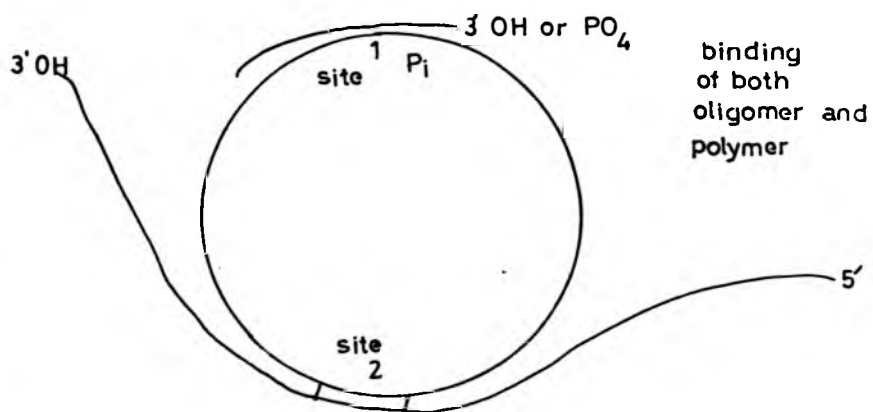
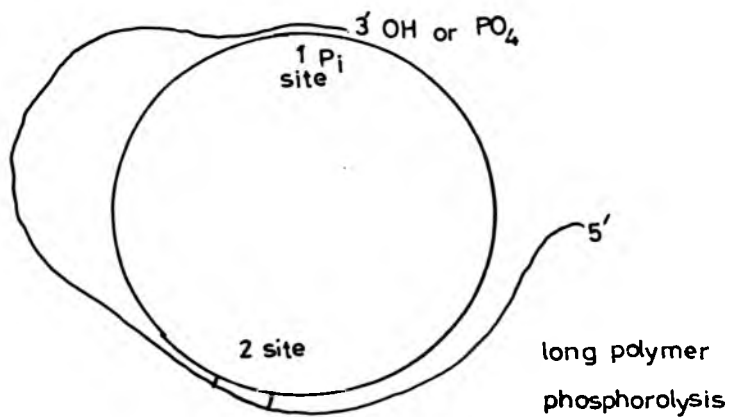
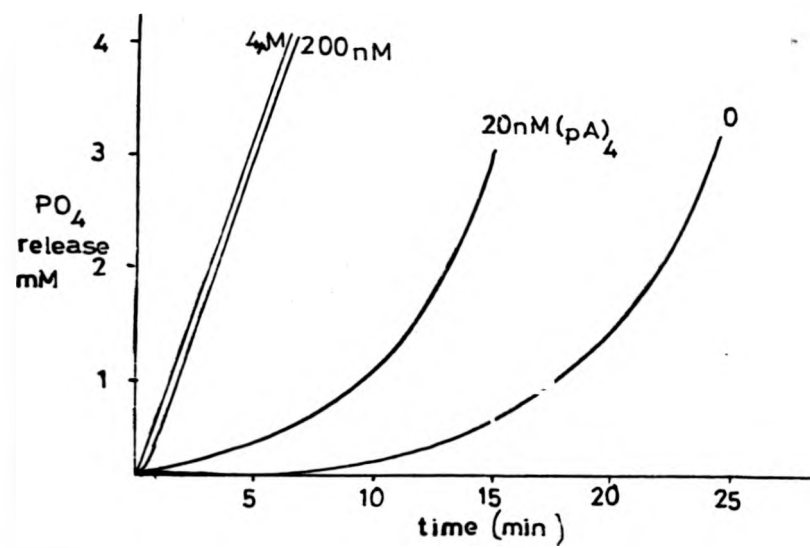


FIGURE 6

Lag phase of polymerisation and the effect of  
primers on PNPase.



distance away, site 2 binds polymer with a very slow dissociation time, stabilises the enzyme substrate complex, and is responsible for the non-synchronous phosphorolysis of attached polymers, (Figure 5). Partially trypsinised *E. coli* PNPase loses affinity for polymer, whilst oligonucleotide binding is unaffected. This provides some indirect evidence for separate binding sites, and the existence of sub-site 2.

#### The polymerisation reaction.

The effect of primers on the polymerisation of nucleotide diphosphates has caused great difficulties in simply interpreting experimental data in terms of active site models and known enzyme kinetics.

The lag phase observed with pure enzyme, (Figure 6), can be abolished by the addition of low concentrations of oligo- or polynucleotides with free 3' hydroxyl groups, whilst in the absence of these molecules, autocatalytic priming often occurs owing to the synthesis of small amounts of oligomer, which eventually stimulate the enzyme to maximal activity.<sup>75</sup> Whether pure PNPases are capable of de novo unprimed synthesis is still unproved. The polymerisation of two nucleotide diphosphates should lead to a polymeric product with a 5'terminal pyrophosphate, which has never been observed. The presence of an endogenous or contaminating nuclease activity has been hypothesised, but no such activity can be detected. However, de novo polymerisation is considered to occur, and proceeds non-synchronously as judged by examination of washings of insolubilised enzyme for intermediate sized polymeric products.<sup>76</sup> The final products tend to be fairly homogenous in size, and stable for lengthy incubation times. One might expect in a freely dissociating system that the average molecular size of the product

would fall with time, but the absence of this effect suggests the polymer is very tightly bound to the enzyme.

Studies of the differential inhibition of the polymerisation and phosphorolysis reactions by dADP in Singer's laboratory<sup>77</sup> provide some evidence for a de novo initiation site on the enzyme. E. coli PNPase phosphorolysis of poly A is inhibited by dADP with a  $K_i$  of 100  $\mu$ M, whilst polymerisation is inhibited with a  $K_i$  of 3-4 mM. This has led to the suggestion that yet another sub-site exists at the enzymes active site, (see Figure 4), which binds both dADP and ADP more tightly than the oligonucleotide primed synthetic site, which is also involved in phosphorolysis.

Primed synthesis is claimed to involve a rapid and complete uptake of primer into polymeric material.<sup>78,79</sup> In conditions of primer excess, a bimodal distribution of product is observed with some oligomer lengthened by only a few nucleotides, whilst other high molecular weight products containing a certain amount of 5' located primer is found. With increasing time, a unimodal distribution of molecular size is found. This suggests that at high oligonucleotide concentrations, competition for the active site can be strong enough to result in the slow synthesis of products which are eventually elongated to also compete with the non-synchronously synthesised long polymer which is also bound at sub-site 2. A final unimodal distribution of product is thus achieved.

Primer dependency is a function of both external conditions and the physical state of the enzyme. Lowering the magnesium concentration, the pH, or addition of inhibitors generally promotes primer dependency, as does partial proteolysis of the enzyme.<sup>10,80</sup> Native E. coli PNPase, when aged, or treated with proteases, becomes entirely primer dependent, whilst primed activity is essentially identical to that of the native enzyme. High molecular

TABLE 2

Priming of E. Coli PNPase.<sup>10</sup>

<u>Substrate</u>	<u>Primer</u>	<u>Ka(μM)</u>
ADP	(pA) <sub>2</sub>	1.2
ADP	(pA) <sub>3</sub>	0.02
ADP	)pA) <sub>4</sub>	0.01
ADP	(pU) <sub>3</sub>	0.3
dADP	(pA) <sub>2</sub>	1100
dADP	(pA) <sub>3</sub>	500
dADP	(pA) <sub>4</sub>	220



weight primers are not effective with these degraded species however. In M. luteus, a similar situation occurs, although some unprimed activity is retained by proteolysed preparations.<sup>81</sup> Treatment with thiols restores the characteristics of the native enzyme, in contrast to the situation with E. coli PNPase, however. Interestingly, primer dependence can be removed by phosphorylation of both M. luteus and E. coli enzymes, using a cAMP-dependent protein kinase.<sup>82</sup>

Attempts to quantify the effects of primers are complicated by the possibility of contaminating oligonucleotides in either enzyme or substrates. The concentration at which a primer half maximally activates polymerisation is known as the half activator constant ( $K_a$ ), and has been investigated with a variety of primers to obtain information about the primer binding site of the enzyme and the mechanism of priming. Some data for the E. coli enzyme are presented in Table 2. The greatly increased affinity for priming in comparison with the binding constants for phosphorolysis is exemplified by (pA)<sub>2</sub>, with the E. coli enzyme, which shows no affinity for the enzyme's phosphorolysis site, whilst its  $K_a$  is  $1.2 \mu\text{M}$ .<sup>83</sup> This is considered to be a consequence of an interaction between the nucleotide diphosphate binding site and the primer binding site. This  $K_a$  is similar to the  $K_m$  for the phosphate exchange reaction, however, which is interpreted as being a result of the full reversibility of the catalytic reaction at the active site, with rapid polymerisation followed by phosphorolysis taking place. In general, the chain length and base composition of the priming oligomer do not have a dramatic effect on priming ability.

Even with the deoxynucleotide containing primer pApAdA, binding

is as tight as with  $(pA)_3$ , although the velocity of the reaction is greatly slowed.<sup>89</sup>

Activation by high molecular weight polymers differs in some respects from oligo A priming, in that a small lag phase can always be demonstrated, which can not be overcome by oligonucleotides at a concentration which would normally cause linear kinetics.<sup>90</sup>

### (3) Structural aspects of PNPase.

The literature reports on the structural characteristics of M. luteus and E. coli PNPase are confused. Although published purification procedures for the PNPases from M. luteus,<sup>91,92,93</sup> E. coli and A. agilis all result in preparations of over 80% purity, as determined by polyacrylamide gel electrophoresis, no amino acid sequence data are presently available, and even molecular weights and subunit structures are open to debate.

Early studies with the E. coli enzyme by Thang suggested a molecular weight of 200,000 daltons for the intact enzyme, whilst equilibrium sedimentation studies in the presence of 8-mercaptoethanol and 8M guanidinium hydrochloride led to the observation of 30,000 molecular weight subunits.<sup>94</sup>

A hexameric structure was proposed on the basis of this evidence which was consistent with electron microscopic data,<sup>95</sup> showing a triangular profile for the enzyme, surrounding a central hole.

Using a different purification procedure, the same authors have provided evidence for a 95,000 dalton subunit by polyacrylamide gel electrophoresis in the presence of 8-mercaptoethanol and 1% SDS, with a minor component (20-50%) of 48,000 molecular weight.<sup>96</sup> Other authors have detected the 95,000 molecular weight component, but not the smaller subunit, using similar techniques.<sup>97</sup>

Yet more conflicting data have recently been presented by Portier et al<sup>98</sup> who have determined the molecular weight of the E. coli enzyme to be 252,000 daltons by gel filtration, (a technique unlikely to be as accurate as ultracentrifugal studies or analytical gel electrophoresis) with constituent subunits of 86,000 daltons. The same authors presented ultracentrifugal and electrophoretic evidence for a molecular weight of 220,000 daltons for the native enzyme shortly before this publication. Another active species of the enzyme, designated form  $\beta$ , produced by the juxtaposition of some steps in his purification protocol, has been characterised by Portier as having a molecular weight of 365,000 daltons. This enzyme form has similar sized major subunits to the other enzyme species (86,000 or 95,000 daltons in size) and also contains smaller 48,000 daltons subunits.<sup>99</sup> The molecular structure of the enzyme is considered to be a trimer of the larger so-called  $\alpha$  subunits, with the form  $\beta$ -enzyme having a number of  $\beta$ -subunits attached. Crosslinking studies, utilising dimethyl suberimidate, are consistent with this interpretation.<sup>100</sup> The function of the  $\beta$ -subunit is unknown, but peptide mapping of the isolated subunits suggest that they are structurally dissimilar, and the  $\beta$ -subunit not a degradation product of the alpha subunits.<sup>101</sup>

The confusion as regards the molecular size and quaternary structure of the PNPase from E. coli may be in part due to proteolytic degradation of the enzyme in the course of purification. Thang has demonstrated the presence of multiple active PNPase bands on polyacrylamide gel electrophoresis by treatment of the enzyme with protease 1.<sup>102</sup> Prolonged storage of the enzyme leads to the appearance of similar higher mobility bands, generally exhibiting a greater primary dependancy, the more degradation has occurred. Such a situation is also observed with the M. luteus PNPase, although the PNPase from this source has been well characterised as existing in two distinct functional and differently sized forms.<sup>103</sup> Form I is the larger, primer-dependent form, with a molecular weight of  $2.7 \times 10^5$  daltons

which is usually associated with a number of smaller active contaminants considered to arise by proteolysis. Form T has a molecular weight of  $2.3 \times 10^5$  daltons, is primer dependent, and may arise from form I by limited tryptic hydrolysis. The most recent investigation of quaternary structure<sup>104</sup> provides electrophoretic data for a 71,000 dalton subunit for form T, whilst additional bands corresponding to about 80,000 and 100,000 daltons were observed with form I. Sedimentation equilibrium studies in 6 M guanidinium hydrochloride, 0.1 M mercaptoethanol with the form I enzyme initially showed a homogenous peak at 100,000 daltons which after forty-eight hours centrifugation, with a variety of speed changes, changed to a position corresponding to 63,000 daltons. Multiple subunit forms, as observed on electrophoresis, could not be detected ultracentrifugally. In the absence of sulphhydryl reagents, only the large subunit could be detected. Sulphydryl groups also appear to have an important functional role in the primer dependence of M. luteus PNPase.<sup>105</sup> Form I may be converted to primer dependence by treatment with N-ethyl maleimide, whilst form T in the presence of dithiothreitol loses its primer requirement. This effect is inhibited by treatment with n-ethyl-maleimide, however, which suggests a free SH group has a role in primer dependency. Trypsinised primer dependent E. coli PNPase does not show such an effect, although primer dependency can be decreased by phosphorylation with a cAMP dependent protein kinase.<sup>82</sup>

(4) Thermophily.

The existence of thermophilic organisms has been known since antiquity, and has been the object of a scientific curiosity since the nineteenth century.<sup>106,107,108</sup> The present view is that thermophiles are the evolutionary precursors of mesophiles and psychrophiles,<sup>109</sup> although the evidence for such a relationship is weak, based on known similarities between the various classes of organisms and the knowledge that the earth in earlier times was hotter than it is now.

Non-photosynthetic bacteria have been observed to grow even in superheated pools at Yellowstone Park, whilst photosynthetic and eukaryotic organisms appear to have definite temperature maxima, above which they are not viable. A plausible explanation for the defined temperature maximum for photosynthetic and eukaryotic organisms is the lability of the complex membrane structures found in these organisms. A number of explanations of thermostability have been proposed, invoking stabilising lipid interactions, rapid resynthesis of denatured molecules, and intrinsic molecular thermostability. Differences in membrane composition are observed between thermophiles and mesophiles and between facultative thermophiles grown at 37° C and 55° C.<sup>110,111</sup> In particular, thermophiles have a higher proportion of branched chain and saturated fatty acids. This, coupled with the observation that alkaline phosphatase of B. stearothermophilus<sup>112</sup> is more stable within the cell membrane than in lysed cells, suggests a role for membranes in thermophily.

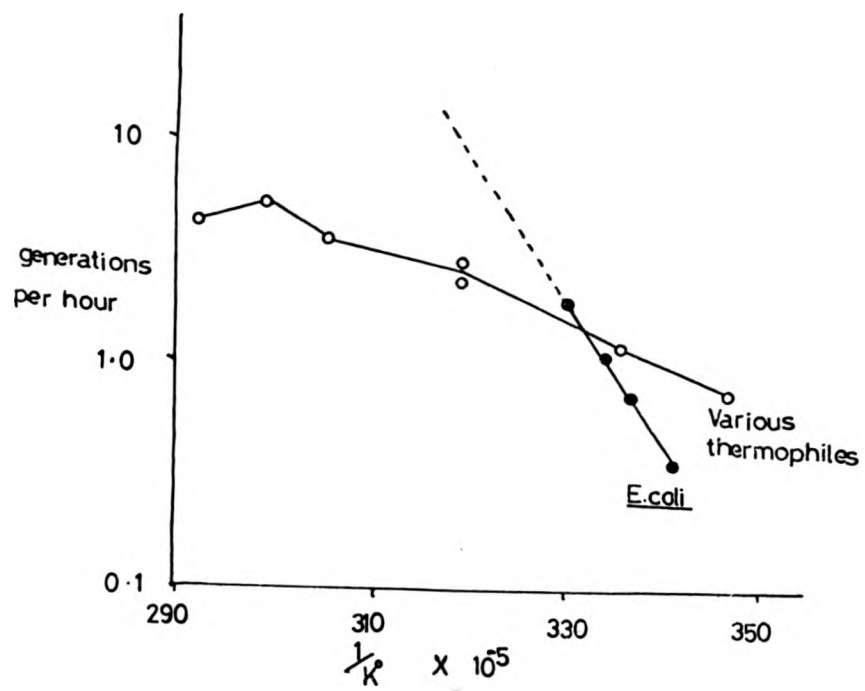
The possibility that rapid resynthesis of degraded molecules could solely account for the ability to grow at high temperatures

has been dismissed by Koffler<sup>117</sup> who pointed out that if this were true, mesophiles should be able to grow at high temperatures and thermophiles at low temperatures. It seems likely that various molecular mechanisms are involved, although an intrinsic thermostability has been observed in a number of proteins purified to homogeneity from thermophilic bacteria.<sup>114</sup> A well-documented example is the  $\alpha$ -amylase (E.C.3.2.1.1.) isolated by Campbell from B. stearothermophilus.<sup>115</sup> which is completely stable at temperatures up to 70°C. Structural studies of the enzyme have revealed that this protein is highly acidic, with a proline content of 15%. Optical rotation studies have revealed little helical content, and these data have led to the suggestion that the enzyme has little secondary structure, and that only imposed by S-S bridges. Unfortunately, other workers have not been able to reproduce this work, and the majority of other proteins studied show closely similar physical properties to their mesophilic counterparts.

There is some evidence to suggest that thermostable enzymes have a more stable tertiary structure, and have sacrificed some efficiency to function at higher temperatures.<sup>116</sup> The aldolase from B. stearothermophilus for instance, when treated with sulphhydryl reagents, is activated at lower temperatures,<sup>117</sup> (Figure 7) whilst it is destabilised at high temperatures. Neither increased numbers of sulphhydryl bridges, or increased hydrophobicity are consistently observed in thermophilic enzymes as compared with mesophiles, although both changes have been observed in a few cases. Nevertheless, many enzymes appear to undergo a conformational change without necessarily losing activity, at temperatures around which hydrophobic interactions may be maximised. This observation has led to the suggestion

FIGURE 7

Arrhenius plot of the growth rate of E. coli and various thermophiles at their temperature optima.<sup>107</sup> The composite slope of the thermophilic organisms is of a lower slope than that for E. coli. This suggests a compromise of metabolic efficiency to enable thermophiles to survive at their temperature optima.





that thermal energy may be absorbed into a hydrophobic cluster, which thus stabilises the active site.<sup>118</sup> T. aquaticus aldolase has been found to be inactive below 58°C,<sup>119</sup> which suggests the necessity for melting a hydrophobic cluster to produce an active enzyme conformation. Such behaviour, as might be expected, has so far only been observed in obligate thermophiles.

Not only structural changes have been observed in the adaption to life at high temperatures. Zuber has demonstrated differences in both enzyme levels and metabolic pathways, using the facultative thermophile B. stearothermophilus under both mesophilic and thermophilic growth conditions.<sup>120</sup> At higher temperatures, the thermal death curves show first order kinetics, suggesting that effects on individual protein molecules are not the limiting factors in determining viability at high temperatures.<sup>121</sup> A likely explanation for this effect is cell membrane lysis.

As judged by published data, we might thus expect PNPases from thermophiles to be physically and catalytically similar to their mesophilic equivalents, with perhaps a lower turnover number than would be expected at their high operating temperatures, but greatly enhanced thermal stability.

5) The aims of the project were primarily to assess the capacity of thermostable PNPsases to synthesise polymers from modified nucleotide diphosphates, and secondarily to elucidate various aspects of the structure and mechanism of the isolated enzymes. The construction of modified polynucleotides is important for the investigation of structure-function relationships in RNA induced interferon production.<sup>122</sup> Interferons are glycoproteins released from cells in response to virus infection, which can in turn induce other cells of the same species to become resistant to virus by selective inhibition of virus transcription or translation.<sup>124</sup> (Figure 8).

The potentially therapeutic uses of highly active and non-toxic interferon inducing molecules, and the requirement for the large scale production of interferon in vitro has led to much work on the structure and mechanism of action of inducing molecules. In vivo, interferon is induced by various micro-organisms, small molecules and polyanions.<sup>125</sup> However, in vitro synthesis seems to be best induced by double stranded RNA. The structural requirements for interferon inducing RNA molecules are presented in Table 3. In an effort to enhance the inducing characteristics of RNA molecules without a concomitant increase in toxicity, various modified RNA molecules have been synthesised and tested as interferon inducers.

Eckstein<sup>58</sup> has modified the phosphate backbone of poly U by preparing homopolymer poly ( $\bar{s}$  U) (Figure 2) from its pyrophosphate analogue, using *E. coli* PNPsase. This polymer forms a double stranded complex with poly A, with a considerably enhanced stability

FIGURE 8

Schematic representation of the induction and  
action of Interferon.

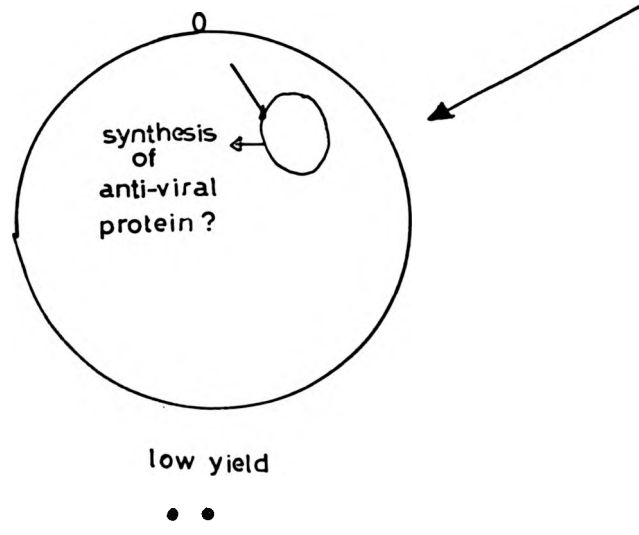
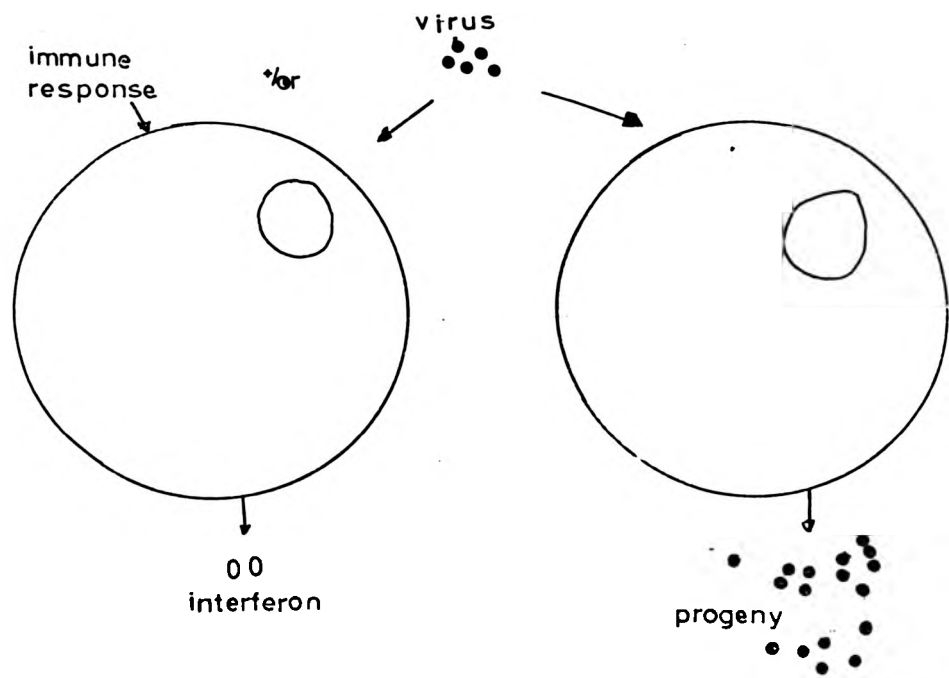


TABLE 3

Structural Requirements for Interferon inducing  
polynucleotides (125)

- (1) High molecular weight ( $> 10^5$  daltons)
  - (2) Ordered stable secondary structure (high  $T_m$ )
  - (3) Presence of 2' hydroxyl groups
  - (4) Resistance to nuclease degradation
-

to nucleases, but unfortunately higher toxicity as well.<sup>126</sup> The therapeutic index was not improved over poly (A) poly (U). Sugar modifications of polynucleotides have also been carried out. Figure 9 summarises the substitutions which have been investigated at the 2' position of nucleotides. Most of these polymers have been synthesised using PNPase from *E. coli*, and manganese as a cofactor, in order to lessen the specificity of the enzyme for the 2' OH group. 2' deoxy 2' amino UDP has been synthesised,<sup>127</sup> but will not form stable double stranded structures for interferon induction studies. Substitution by halogens at the 2' position has been investigated by Eckstein amongst others.<sup>128</sup> Chloro substitution results in a uracil polymer with markedly different and enhanced base stacking compared to poly rU, whereas the same substitution in CDP seems to have little effect on secondary structure.<sup>129</sup> Poly (2' fluoro U) forms a more stable hybrid with poly A than poly U, although the polymer alone exhibits no higher structure at temperatures above 2.5°C. Unfortunately, none of these sugar modifications result in active interferon inducing molecules.

Base modifications, on the other hand, have been investigated in some detail and do not usually abolish interferon inducing capacity. Michelson<sup>130</sup> pioneered the synthesis of modified polynucleotides, polymerising uridine diphosphates, substituted at their 5 position with halogens or hydroxyl groups. He also synthesised poly (i<sup>5</sup>C) by similar methods.<sup>131</sup> The substituted polymers showed differing stabilities of helix formation with poly A, the order of stability being IodoU > BrU > ClU > U > FU, which is consistent with the notion that poorly understood base-stacking interactions rather than hydrogen bonding are responsible

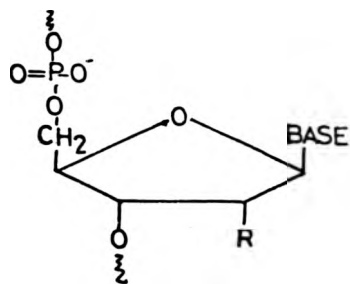
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FIGURE 9

2' substituted nucleotide diphosphates, and the structure of 8, 2'-anhydro-8 mercapto-9-B-D-arabinofuranosyladenine 5' diphosphate (A<sup>S</sup>DP).

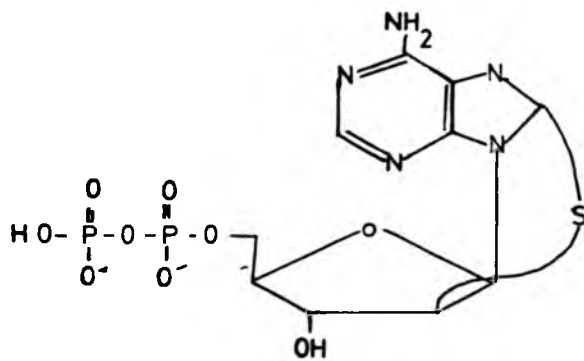




R= OMe  
 OEt  
 OCOCH

N  
 NH<sub>2</sub>

Cl  
 F



for helix stability. Other pyrimidine modifications have included the synthesis of 5 hydroxy,<sup>132</sup> methyl and chloro C, the last two being good interferon inducers, whilst poly (5-hydroxycytidylic acid) does not appear to form duplex structures, but exerts an anti-viral effect by a non-interferon mediated mechanism.<sup>133</sup> 6- substituted pyrimidines are very poor substrates for polynucleotide phosphorylases, but poly (6 aza C) has been synthesised in poor yield,<sup>134</sup> and poly (6 acetyl C) has been synthesised by direct reaction with the polymer.<sup>135</sup> Neither polymer forms stable duplexes with poly I however, and they are consequently poor interferon inducers.

Purine modifications have mainly been confined to substitution at the 8 position, as hydrogen bonding properties are effected by substitution at most other positions. Bulky substituents there,<sup>136</sup> as well as synthesis of poly (2 methyl 1)<sup>137</sup> and poly (2 methylamino A)<sup>138</sup> have led to polymers which show poor helix formation. We are thus left with poly(rI) poly (rC) and poly (rG) poly (rC) as the best available interferon inducers, although both are too toxic for effective therapeutic use. The synthesis of more modified polynucleotides is still of interest, however, in order to elucidate those features of the receptor for double stranded RNA which might allow a rational synthesis of an inducer of high therapeutic index. Modified polynucleotides which can act as photoaffinity probes, such as poly (8 azido A)<sup>139</sup>, and toxic polymers which may play a useful part in the treatment of external viraemias, such as poly (5 hydroxy C)<sup>133</sup> are also worthy of investigation. The use of thermostable PNPases at elevated temperatures might be expected to further such studies by allowing poor substrates to be polymerised both faster and in better yield, as unfavourable steric interactions in modified substrates are decreased with higher temperature.

## CHAPTER TWO

### 1) Introduction.

Since the date of discovery, contradictory reports on both structural and mechanistic aspects of E.coli PNPase have appeared, even from identical laboratories.<sup>10,98</sup> A possible source of such discrepancies appeared to be the then generally used purification procedure of Williams et al.,<sup>83</sup> which involved cell breakage by mechanical shearing and protamine sulphate precipitation. Preparations not treated with protamine, which is often contaminated with proteases,<sup>72</sup> showed different molecular weight characteristics from treated preparations. Furthermore, new methods in protein chemistry such as the development of effective general techniques for biospecific affinity chromatography<sup>73</sup> have become available since Williams developed his purification. We decided to develop a new purification procedure in order to obtain an enzymic activity with reproducible characteristics, to further the investigation of structure-function relationships in the induction of interferon by synthetic polynucleotides. Having developed such a procedure, we then applied it to the purification of PNPases from other organisms. The facultative thermophile B. stearotherophilus, and the obligate thermophile, T. aquaticus, are both viable at temperatures of 70°C. As PNPase activity seems so universal in the prokaryotic world, we chose these organisms in the hope that their PNPases would be intrinsically thermostable, as might be expected from studies with other thermophilic enzymes. Considerations of reaction rate were not as significant in this choice as the possibility of inducing poor substrates for mesophilic PNPases to assume more suitable configurations and facilitate their polymerisation. As mentioned in the previous chapter, bulky substituents in the purine ring at the 8-position, or in pyrimidines at

the 6-position, result in a higher proportion of the molecules assuming the syn configuration, presumably due to steric considerations. Molecules in the syn conformation are poor substrates for PNases, although they bind more strongly to the active site. We hoped that, at higher temperatures, such a transition might be easier, and we would thus be able to polymerise modified nucleotide diphosphates which were competitive inhibitors at the operating temperatures of mesophilic enzymes.

## 2) Materials and methods.

Radiochemicals were purchased from the Radiochemical Centre, Amersham. Nucleotide diphosphates and alkaline phosphatase (EC 3.1.3.1) were obtained from Boehringer Mannheim GmbH. Polynucleotides were obtained from PL Biochemicals Inc. Ribonucleases and deoxyribonucleases were obtained from BDH. Oligo(dT)sepharose was the gift of J.C. Smith, Searle Research Laboratories, or was synthesised as described in the text. Gel filtration media were obtained from Pharmacia or Bio-rad laboratories. Ion exchange Sepharoses were purchased from Pharmacia, and ion-exchange celluloses from Bio-rad laboratories. Freeze-dried cell pastes of E. coli B, B. stearothermophilus (NCIB 8924) and T. aquaticus were obtained from MRE Porton. All other materials were obtained from standard laboratory sources and were of Analar grade unless otherwise stated.

### Assay systems.

Protein was assayed by the Biuret or Folin-Lowry methods,<sup>84</sup> Purified proteins were occasionally assayed spectrophotometrically,<sup>85</sup> using the formula  $1.55 \text{ OD}_{280} - 0.76 \text{ OD}_{260} = \text{Protein concentration}$  in mg/ml.

### PNPase assays.

#### Polymerisation assays.

a) PNPase was assayed by the colorimetric determination of phosphate released with time. This assay is as sensitive as the NADH linked phosphorolysis assay, but is not continuous, and is more susceptible to interference by contaminating enzymic activities such as phosphatases. Its simplicity and cheapness commend it for use with

partially purified preparations. The assay mixture contained 50 mM Tris.HCl pH 9.2, 20 mM ADP, 7.5mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, 0.6 M KCl and 100 μM oligo A primer. Phosphate release was monitored by the method of Chen et al.,<sup>86</sup> after removal of protein by the addition of TCA to 5% followed by bench centrifugation. To an aliquot of 0.1 ml of supernatant, 2.5 ml of 0.42% ammonium molybdate in 1 N HCl : 10% ascorbic acid (6.1 v/v) was added, and the sample incubated at 50°C for ten minutes. Absorbance at 720 nm was measured on an SP 500 Unicam spectrophotometer.

b) An assay less susceptible to interference by endogenous phosphate was also used.<sup>87</sup> [<sup>14</sup>C] ADP was incubated under the conditions described above. After stopping the reaction with glacial acetic acid, an aliquot from the reaction mixture was chromatographed on Whatman 3 mm paper in ethanol : 1 M ammonium acetate (1:1 v/v). Activity was computed by measuring the counts at the origin (polymer) and those co-eluting with ADP.

c) An NADH linked spectrophotometric assay first developed by Ochoa<sup>88</sup> was carried out according to Godefroy.<sup>90</sup> The reaction mixture contained in 0.4 mls, 50 mM Tris.HCl pH 8.2, 10 mM potassium dihydrogen orthophosphate, 4.6 mM magnesium chloride, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 50 μg/ml Pyruvate Kinase (E.C. 2.7.1.40), 50 μg/ml Lactate Dehydrogenase, (E.C. 1.1.1.27), and about 0.3 units of PNPase, as well as any substrates or inhibitors. The magnesium was added last to avoid the slow precipitation of magnesium phosphate, and the reaction followed at 366 nm, taking continuous readings at intervals of a few seconds, and using an extinction coefficient of 3400 for NADH to compute the amount of ADP released. This assay has the great advantage of being continuous when linked to a chart recorder from a recording spectrophotometer.

d) Radioactive phosphorus can be separated from ADP by a addition of a precipitating reagent described by Sugino and Miyoshi.<sup>99</sup> This forms the basis for a radio-assay described by Klee,<sup>9</sup> and modified here where the course of the phosphorolysis reaction is followed by the uptake of radioactive phosphorus into nucleotide diphosphates. The incubation mixture contained 50 mM Tris.HCl pH 8.2, 1 mM DTT, 1 mM EDTA, 0.6 M KCl, 100  $\mu$ M poly A and 10 mM [<sup>32</sup>P] labelled  $\text{KH}_2\text{PO}_4$  with an activity of about  $10^5$  cpm. The reaction was stopped by the addition of 3 ml of freshly made precipitating reagent containing 5 mM triethylamine-HCl pH 5.0, 64 mM ammonium molybdate, 160 mM perchloric acid, as well as 0.2% SDS. Activities were calculated using a blank which had enzyme added after the precipitating reagent, and a standard to which 3 mls of water was added. After centrifugation, an aliquot from the supernatant of each tube was counted in a scintillation fluid comprising 5.5 g PPO, 100 mg POPOP, 330 ml Triton X 100 and 667 ml toluene.

Activity was computed by the relationship

$$\frac{\text{cpm sample} - \text{cpm blank}}{\text{cpm standard} - \text{cpm blank}} \times 100 = \% \text{ phosphate present converted to nucleotide diphosphate}$$

using corrected counts and carrier free [<sup>32</sup>P]. This assay is specific and reproducible, and the method of choice for the assay of impure PNPase preparations.

#### In situ PNPase assay.

The activity stain developed by Thang<sup>140</sup> for locating PNPase on acrylamide gels after non-denaturing electrophoresis was carried out according to Klee.<sup>9</sup> A control gel containing enzyme, but not incubated with nucleotide diphosphate was also stained to remove the possibility of endogenous nucleic acids being mistaken for PNPase activity. The assay utilises acridine orange to detect polynucleotide synthesised on incubation of the gel with 20 mM ADP, 7.5 mM  $\text{MgCl}_2$ , 50 mM Tris.HCl pH 9.0 .

### Assay of contaminating enzyme activities.

As an indicator of the state of contamination of purified PNPase ribonuclease, adenylyl kinase and phosphatase activities were assessed. These assays were carried out as described in reference 141.

### Purification procedures investigated.

E. coli, B. stearothermophilus or T. aquaticus were all directly suspended in buffer comprising Tris-HCl pH 8.2, 1 mM DTT, 1 mM EDTA, either directly from frozen cell pastes obtained commercially or after harvesting from freshly harvested cells. B. stearothermophilus was grown under the conditions described by Sargent<sup>142</sup>, E. coli under the conditions described by Werznan<sup>143</sup> and T. aquaticus according to Brock and Freeze<sup>144</sup>, with the exception of the substitution of tris(hydroxymethyl) aminomethane for nitrilotriacetic acid.

Cells were generally lysed at a concentration of about 20g per 100 ml of buffer, using lysozyme/EDTA treatment<sup>145</sup>, or by ultrasonication for T. aquaticus. After lysis, cell debris was centrifuged off, the pellet washed, and the washings combined with the previous supernatant before purification was attempted.

A variety of purification procedures were investigated for their potential use in the preparation of pure PNPase. The methods used are detailed below.

#### 1) Ammonium sulphate precipitation.

The solubilities of PNPases from E. coli, B. stearothermophilus and T. aquaticus were investigated in various concentrations of ammonium sulphate, using a saturated ammonium sulphate solution rather than solid ammonium sulphate adjusted to pH 7 at 4°C added slowly to the required concentration.

#### 2) Heat denaturation.

An early literature report suggested that E. coli PNPase was relatively thermostable.<sup>146</sup> Diagram 2.1 shows the stability of E. coli PNPase in

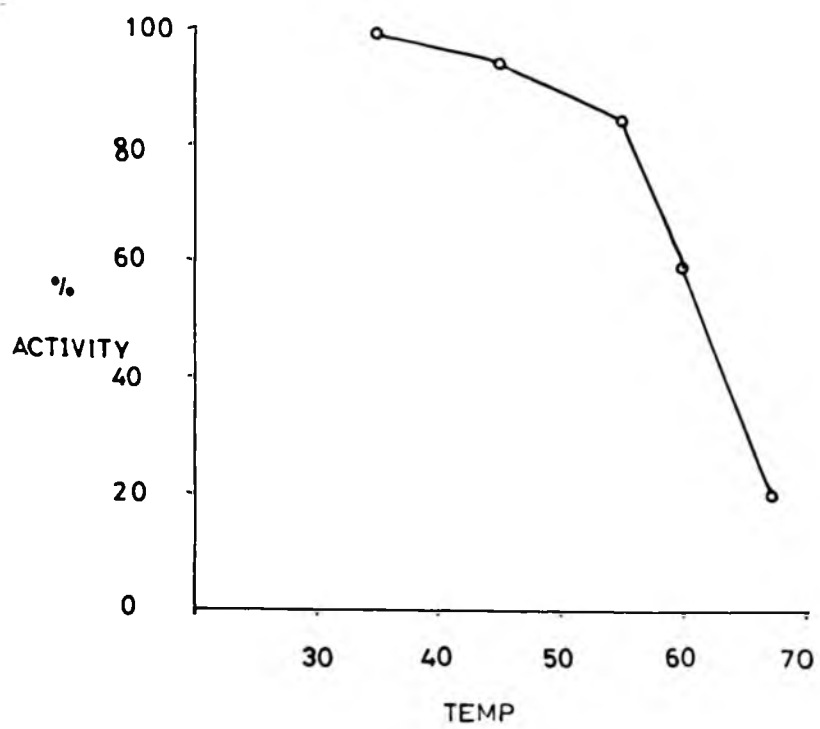
FIGURE 2.1

The residual E. coli PNase activity after seven minutes incubation at the stated temperature.



FIGURE 2.1

The residual E. coli PNPase activity after seven minutes incubation at the stated temperature.



50 mM Tris pH 8.2, 1 mM DTT, 1 mM EDTA, 0.4 M KCl to heat. On the basis of these observations, a heat denaturation step was incorporated into the purification procedure of the *E. coli* enzyme.

3) Preparative polyacrylamide and agarose gel electrophoresis.

Preparative gel electrophoresis was carried out on G-25 Sephadex according to Virella,<sup>147</sup> or on polyacrylamide according to Griffiths.<sup>148</sup> Medium grade G-25 Sephadex was equilibrated with barbital/boric acid buffer, poured on to a perspex slab, 30 x 10 x 0.5 cm., and buffer saturated Whatman 3 mm paper wicks were applied to the ends of the plate. The gel was partially dried by pre-electrophoresis at 40 ma, then a trough was carved at the cathodic end, into which sample in 0.5% bromophenol blue and electrophoresis buffer was applied.

After electrophoresis, sections of the gel were removed and washed in 50 mM Tris-HCl pH 8.0, and the washings assayed for PNPase activity and protein.

Acrylamide gel electrophoresis was carried out on 5% gels prepared according to Weber.<sup>149</sup> After electrophoresis, a longitudinal slice was stained for protein in Coomassie brilliant blue R 250, and those sections of the gel containing protein were isolated by slicing with a razor blade. These slices were macerated in 50 mM Tris-HCl pH 8.0, then stored at 4°C overnight. The eluates were then assayed for PNPase activity.

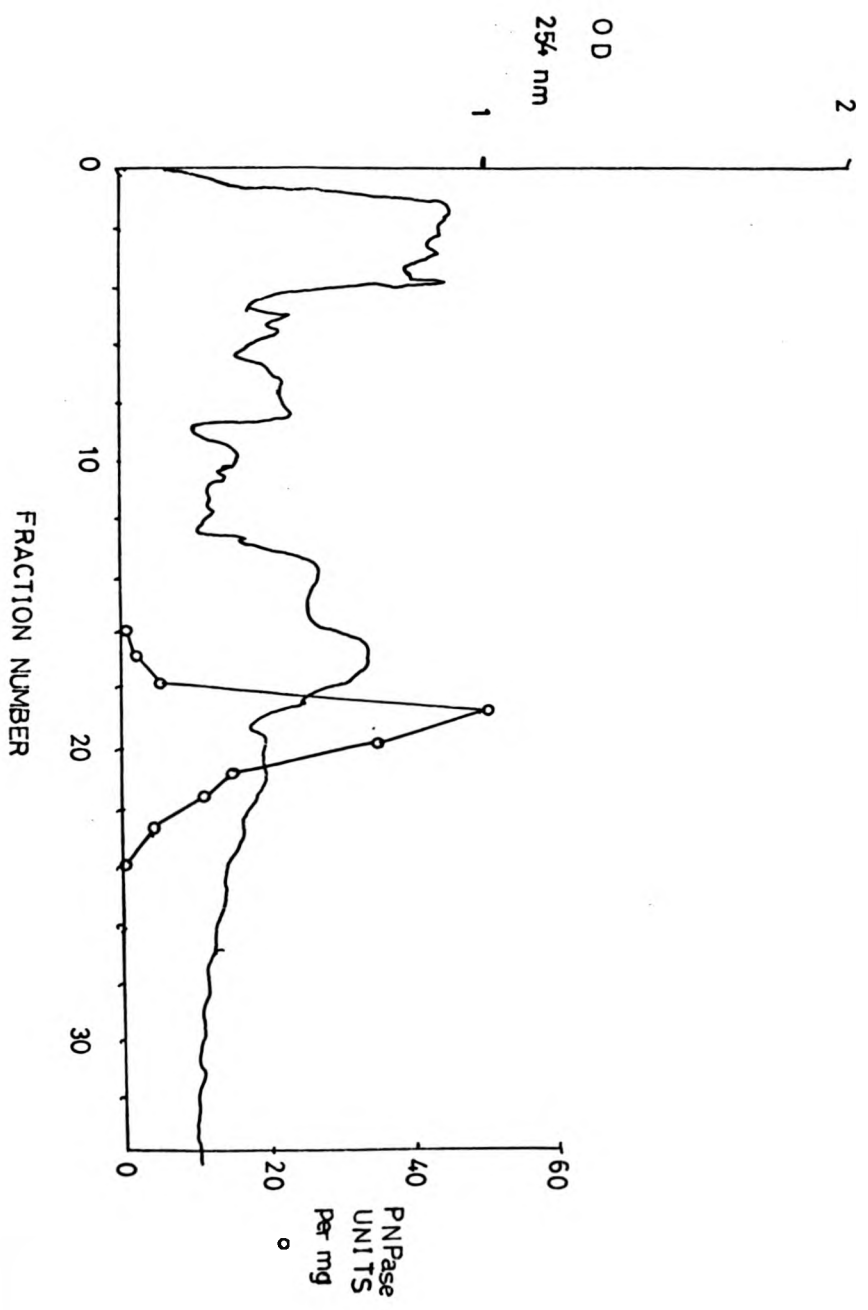
Figure 2.2 shows the elution profile of an *E. coli* cell extract electrophoresed on a 10 x 2.5 cm column gel of G-25 Sephadex, otherwise under the conditions described above. The eluate from the anodic end of the gel was passed through an LKB unicord monitor and fractions collected. The potential of this purification method can be clearly seen.

FIGURE 2.2

LKB Uvicord trace of E. coli eluate after  
G-25 Sephadex electrophoresis.

Protein = —

Activity = o



4) Gel exclusion chromatography.

Sephadex G 200, LKB ultrogel Aca 34 and Bio-gel A 0.5 M were used according to the maker's instructions. All chromatography was carried out at 4°C in Tris-HCl(50 mM)pH 8.2, 1 mM DTT, 1 mM EDTA.

5) Ion exchange chromatography.

DEAE-cellulose, TEAE-cellulose and DEAE-Sephadex (A 25 and A 50 grades) were assessed for purification purposes, using linear increasing salt gradients after sample application at 4°C. Column equilibration was routinely assayed by measuring the pH of the eluate until it was the same as the washing buffer, and the salt concentrations at which samples eluted were measured by refractometer.

6) Affinity chromatography.

A variety of ribo- and deoxy-ribo nucleotide substrates and inhibitors of PNPases were immobilised by attachment to Sepharose 4B and used as chromatographic media. Chromatography on affinity columns was carried out in 50 mM Tris-HCl pH 8.2, 1 mM DTT, 1 mM EDTA, 10 mM MgCl<sub>2</sub>, and elution was effected by linear increasing salt gradients.

The general method of insolubilisation was to use cyanogen bromide activated Sepharose prepared according to Poonian et al.<sup>150</sup> Cyanogen bromide (250 mg/ml of gel) was added in powdered form in one batch to a stirred slurry of Sepharose 4B in water. Sodium hydroxide (5M) was added to the slurry to maintain the pH at 11 until the pH no longer fell, at which time the activation was considered to be complete. The Sepharose was then washed on a filter with several volumes of ice cold potassium phosphate buffer (50 mM pH 8.0) then the nucleic acid to be bound added (about 10 OD 260 units per ml) and the mixture stirred at 4°C for 24 hours. A column of the material was then packed and washed with several volumes of potassium phosphate, 50 mM pH 8.0

then with the same buffer containing 1 M potassium chloride, until no more material absorbing at 260 nm was eluted. This method was used to bind poly rA and poly rU to Sepharose. ADP was bound to Sepharose by a periodate linkage method, using adipic acid dihydrazide as a spacer.<sup>151</sup>

ADP (4 mg/ml) in 0.1 M sodium acetate pH 5.0 was stirred at room temperature for one hour in the presence of 10 mM magnesium chloride, 1 mM sodium periodate.

Cyanogen bromide activated sepharose, prepared as described above, was treated with adipic acid dihydrazide, and the oxidised ADP, 50  $\mu$ g/ml of gel, added in 0.2 M sodium acetate pH 5 and stirred gently at 4°C overnight. The reacted gel was then washed in salt (1 M KCl) until no unreacted ADP was eluted.

#### Synthesis of oligo-(dT)-Sepharose.<sup>152</sup>

Thymidine monophosphate (4 mM) was made anhydrous by dissolution in pyridine (25 ml) followed by evaporation under reduced pressure. This was repeated five times. The gum was then dissolved in dry pyridine (30 ml) and DCCD (4.2 gm) added. The mixture was shaken at room temperature for five days. Water (25 ml) was then added, and after three hours the solution was ether extracted (6 x 100 ml). The aqueous layer was then concentrated in vacuo to a syrup which was dissolved in water (50 ml), and the pH adjusted to eight with lithium hydroxide. The solution was filtered from a small quantity of dicyclohexyl urea, and made up to 100 ml with water. The solution was then applied to a DEAE-cellulose column ( $\text{HCO}_3^-$  form, 3 x 30 cm) which was washed with water (300 ml). A linear gradient (500 ml) of triethylammonium bicarbonate (pH 7.5, 0 - 0.45 M) was applied, and oligo-(dT) of increasing chain length eluted. Oligo-(dT) of ~ 2 to 20 residues in length was pooled and evaporated to dryness prior to binding to

Sepharose. Oligo(dT) (100 mg) was dissolved in dry pyridine (10 ml) and the solution evaporates in vacuo. This procedure was repeated, then recrystallised p-nitrophenol (600 mg) was dissolved with the oligo(dT) in dry pyridine (10 ml) which was again evaporated to dryness in vacuo. The residue was suspended in dry pyridine (10 ml) to which was added DCCD (800 mg). The mixture was stirred for 72 hours in a stoppered flask at room temperature in the dark. The pyridine was removed under reduced pressure, and the residue dissolved in water and extracted several times with ether. The aqueous layer was then evaporated to dryness.

The residue was now dissolved in 50% aqueous methanol (0.5 - 1 ml) and palladium on charcoal (50 mg) added. Reduction was carried out, using hydrogen at 35 psi for two hours in a Parr bomb. The catalyst was removed by bench centrifugation, and the supernatant evaporated to dryness. The p-aminophenyl oligo(dT) was then bound to cyanogen bromide activated Sepharose as described above.

#### DNA cellulose chromatography.

Double and single stranded DNA and RNA agarose gels were prepared according to Schaller et al.<sup>153</sup> Calf thymus DNA or Torula yeast RNA at a concentration of about 20 mg/ml in 0.02 M Tris; 0.2 M magnesium acetate pH 7.4, or in 0.02 M sodium hydroxide (single stranded DNA) was mixed at 60°C with 16% agarose, then cooled rapidly in ice. The gel was then cut and sieved using an 80 mesh sieve suspended in 100 mM Tris-HCl pH 8.0 magnesium chloride 10 mM, 1 mM DTT, and packed in a column 10 x 1 ml. The column was then washed in 1 M KCl until no more OD 260 absorbing eluate could be observed.

#### 7) Miscellaneous chromatographic methods.

Hydroxylapatite chromatography gave good but inconsistent results.<sup>154</sup> Phosphocellulose chromatography was also investigated and found to give reasonable purifications.<sup>155</sup> Other methods tried were the use of insolubilised antibody columns, bound to cyanogen bromide Sepharose



which gave poor results, probably due to the crude enzyme used to raise antisera, and continuous sucrose density gradient centrifugation, using 5 - 20% sucrose gradients. Both these techniques are more fully dealt with in the next chapter, in relation to the structural properties of the various enzymes.

Material was applied to the unsolubilised antibody column in Tris-HCl 50 mM pH 8.0, 1 mM DTT, 1 mM EDTA and eluted by an increasing linear salt and pH gradient (pH 8.0 to pH 10.0, 0 - 2 M KCl). Concentration and desalting of samples were carried out in a Bio-rad hollow fibre device (with a nominal molecular weight cut-off of 5000) according to the makers' instructions. No loss of activity occurred with these devices. Preparation of oligo-(rA) primers, for the assessment of primer dependency, was carried out according to reference <sup>141</sup>.

### Results.

#### 1) Sources of PNPase.

Amongst the organisms screened for PNPase content, in order to facilitate the preparation of large quantities of material were Bacillus subtilis, Saccaromyces cerevisiae, B. stearothermophilus and T. aquaticus. The amount of PNPase in phosphorolysis units per mg is presented in table 2.1 where all the organisms were harvested in late logarithmic growth phase.

Early observations of the specific activity of PNPase through the growth cycle of E. coli showed that about twice as much activity per mg of protein was present in the early logarithmic phase of growth.<sup>4</sup> B. stearothermophilus and T. aquaticus were screened for PNPase content throughout the growth cycle. As shown in figure 2.3, the enzyme activity for B. stearothermophilus PNPase varied in an analogous fashion to that observed with E. coli. We used the phosphorolysis assay however, whilst Grunberg-Manago used a phosphate release assay. Similar results were obtained with T. aquaticus. The increase in specific

FIGURE 2.3

Variation of cellular content of PNase through  
the growth cycle of B. stearothermophilus.

Turbidity = ○

Specific activity = ●

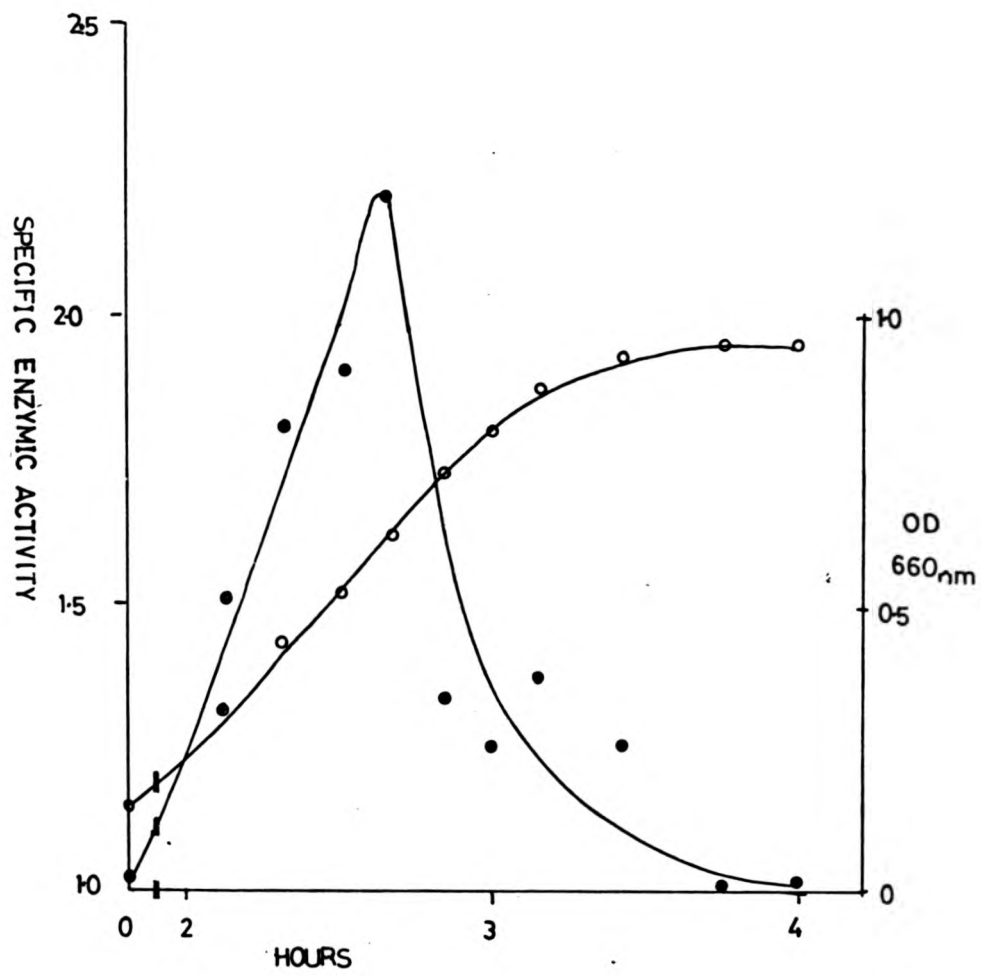


TABLE 2.1

<u>Organism</u>	<u>PNPase content in phosphorolysis units/mg protein</u>
E. coli	0.7
B. stearothermophilus	1.2
T. aquaticus	0.2
B. subtilis (NCIB 9590)	0.9
S. cerevisiae	0.00

---

TABLE 2.2

Preparation of E. coli PNPase

	Units <sup>*</sup> /mg	Purification	Yield
Lysozyme/EDTA lysed cell extract	0.7	1	100
Heat treated at 50°C for 7 minutes	3.6	5.1	90
40 - 60% ammonium sulphate cut	12.8	18.3	73
TEAE-cellulose eluate	63	90	65
DEAE 50-Sephadex column	235	336	58
LKB Ultrogel Aca 34 column	419	599	55

\* Assays carried out at 37°C

TABLE 2.3

Purification of *B. stearothermophilus* PNPase

<u>Step</u>	<u>Units/mg</u>	<u>Purification</u>	<u>Yield</u>
Lysozyme/EDTA lysed cell extract	1.2	1	100%
33 - 55% ammonium sulphate cut	6.5	5	82%
TEAE cellulose column	59	49	74%
DEAE-sephadex A 50 column	283	203	41%
Aca 34 Ultrogel column	603	500	41%

TABLE 2.4

Purification of *T. aquaticus* PNPase

<u>Step</u>	<u>Specific activity</u>	<u>Purification</u>	<u>Yield</u>
Sonicated cell extract	0.8	1	100%
35 - 55% ammonium sulphate cut	4	5	57%
TEAE-cellulose column	25	31	21%
DEAE-sephadex A 50 column	172	215	7%

A full description of the purification procedures used is to be found in reference 141, which is enclosed at the back of the thesis. Throughout the thesis, units of enzyme activity are quoted in phosphorolysis units, measured by assay procedure, except where otherwise indicated.

TABLE 2.5

<u>Purification procedure</u>	<u>Range of purification attained</u>
DEAE and TEAE celluloses	5 - 20 fold
DEAE sephadexes	5 - 20 fold
gel filtration media	3 - 10 fold
electrophoresis on G 25 sephadex	20 - 30 fold
ammonium sulphate precipitation	2 - 6 fold
HAP chromatography	0 - 6 fold
phosphocellulose	3 - 8 fold
agarose trapped DNA and RNA	0 fold
Poly-A sepharose	0 - 120 fold
oligo(dT) sepharose	0 - 120 fold
ADP-sepharose	0

---

activity of the enzyme through the early part of the growth cycle was not reason enough to harvest cells in early log phase for the purposes of the purification procedure however, as cell density at this stage was low.

The results for the purification procedures developed for E. coli, B. stearothermophilus and T. aquaticus PNPases are presented in tables 2.2, 2.3 and 2.4 respectively. Table 2.5 shows the range of purification achieved with various purification procedures. Similar results were obtained for all three enzymes. It is of interest to note that the elution profiles of all three enzymes on gel filtration were identical in the presence and absence of blue dextran, suggesting the absence of any 'dinucleotide loop' region in these molecules.<sup>156</sup>

Throughout the early stages of the purifications, all the enzymes exhibited primer independency in the polymerisation reaction, which was maintained if A 25 Sephadex was used, but lost when A 50 Sephadex was the medium employed. However, leading and trailing edges of the A 50 Sephadex eluate showed differing primer dependencies. This suggests that a factor conferring primer-independency was lost from the enzyme during this chromatographic step. That the putative factor was not lost on TEAE-cellulose or A 25 Sephadex chromatography suggests that such a factor would have a similar charge to that of the PNPase with which it was associated, but a different molecular weight, as not only A 50 Sephadex chromatography, but also gel filtration on Aca 34 Ultrogel after A 25 Sephadex chromatography made the PNPase primer dependent. The differing behaviour on A 25 and A 50 Sephadex suggests that such a factor would have a molecular weight of  $5 - 40 \times 10^3$  daltons. Reconstitution experiments were unsuccessful however, using various eluted fractions from A 50 Sephadex columns with primer dependent enzyme, although those fractions rich in material absorbing at 260 nm showed a small effect. This inability to restore primer

independency could be the result of the dilution of the factor as it was eluted from the column. The ability to produce primer dependency by gel filtration suggests that such a factor would be rather weakly associated with the enzyme, but it is interesting to note that gel filtration of unpurified PNPase preparations does not lead to primer dependency. One might imagine that the association of the factor with PNPase is stabilised by the presence of nucleic acids, with which crude PNPases are usually found to be associated, and that the removal of the bulk of nucleic acids by TEAE and DEAE-Sephadex chromatography labilises the complex. There is no direct evidence for such a factor however, and until reconstitution of primer independency can be achieved, its existence must be considered mere speculation. The recent report of two differing sized PNPases in E. coli by Portier<sup>98</sup> is of some interest in this respect, as he has identified a non-catalytic subunit of approximately 48,000 daltons and unknown function. Unfortunately, no description of variation in primer dependency between the two forms of PNPases has been made by Portier. However, Portier claims that one form of the enzyme has a molecular weight consistent with the presence of two 48,000 dalton subunits.

Affinity methods of chromatographically purifying PNPases were abandoned when it became apparent that conventional protein separation techniques would simply and adequately produce reasonable yields of PNPases. Although insolubilised nucleic acid columns were potentially effective for purification purposes, their low capacity and irreproducibility with crude PNPase preparations made them less attractive. The most successful method tried was that developed by Smith,<sup>152</sup> who claimed to have purified B. stearothermophilus PNPase

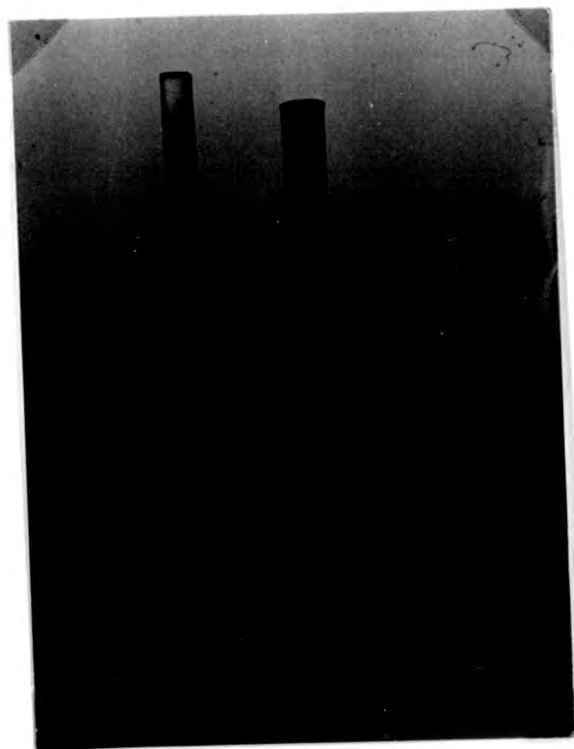


to homogeneity by oligo(dT) Sepharose chromatography. Although this preparation was reproducible in our laboratory, we found that eluted enzyme from the (dT) Sepharose column was heavily contaminated with OD 260 absorbing material (ratio 260 : 280 > 1) and that on gel electrophoresis, even under non-denaturing conditions, more than one protein band could be distinguished. Monitoring of homogeneity was carried out throughout the purification procedures by polyacrylamide gel electrophoresis. Plates 1 and 2 show the progress of B. stearothermophilus from a crude cell extract to apparent homogeneity. Plate 3 compares preparations by conventional purification techniques, and by the affinity method of Smith. Distinct bands can be distinguished in the latter preparation on non-denaturing polyacrylamide gel electrophoresis. Apart from polyacrylamide gel electrophoresis, isoelectric focussing was also used as a criterion for homogeneity. The system described by O'Farrell<sup>157</sup> was used, and plate 4 shows a stained focussed gel after washing out residual ampholines of B. stearothermophilus PNase.

Plate 1

Polyacrylamide gel electrophoresis of

- (A) Whole cell extract of B. stearothermophilus on  
7.5% gels (with SDS)
- (B) TEAE cellulose eluate of cell extract in 7.5% gels



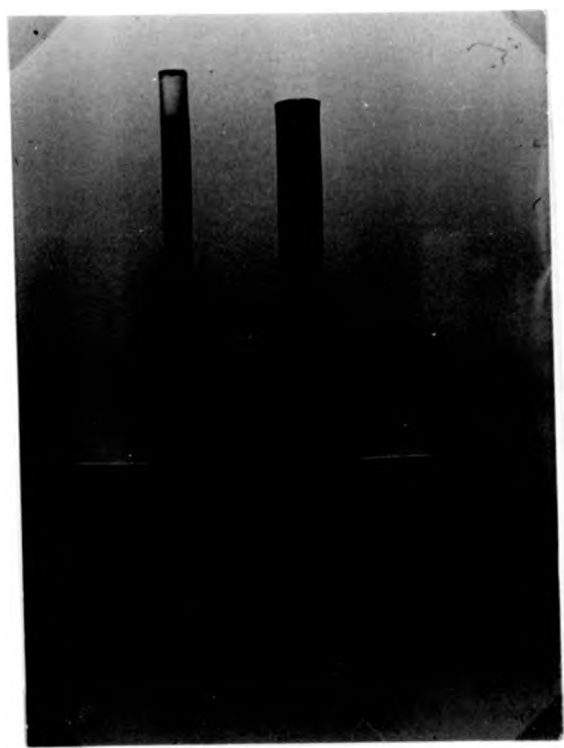
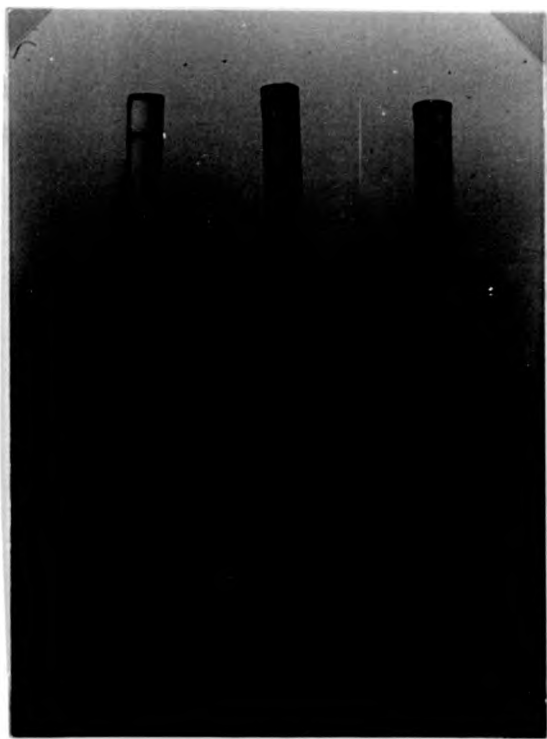


Plate 2

Polyacrylamide gel electrophoresis of B. stearothermophilus  
PNPase in 7.5% gels using

- (C) Tris-Glycine
- (D) Phosphate
- (E) Borate/acetate buffer systems



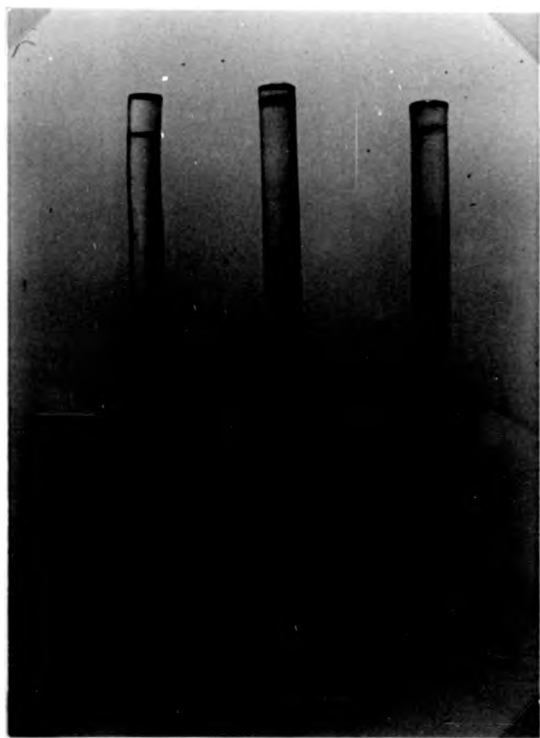
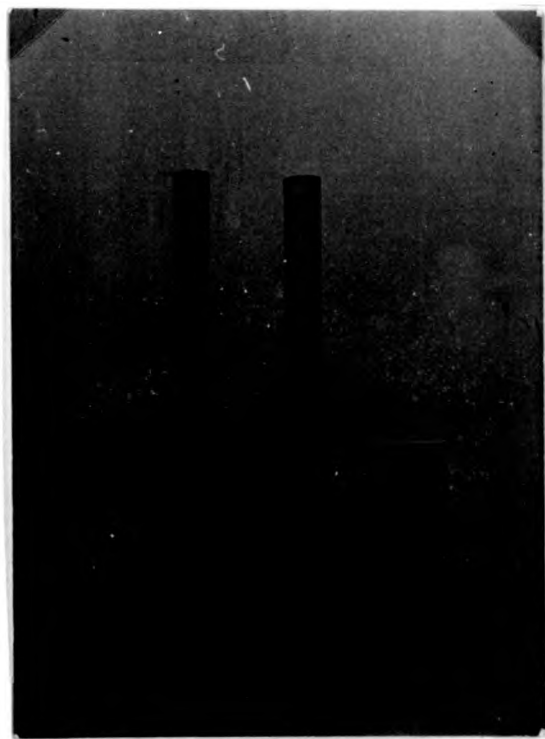


Plate 3

Polyacrylamide gel electrophoresis in 7.5% gels using a phosphate buffer system of

- (D) B. stearotherophilus PNPase prepared according to the text
- (F) B. stearotherophilus PNPase prepared by chromatography on oligo(dT)sepharose





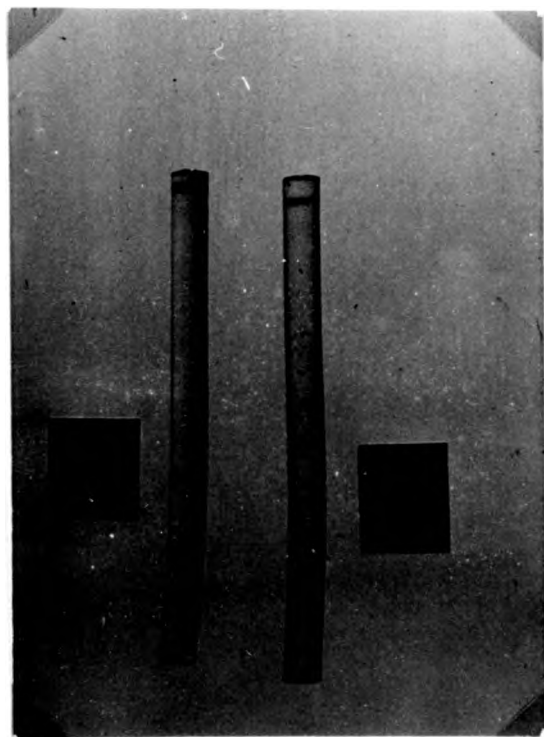
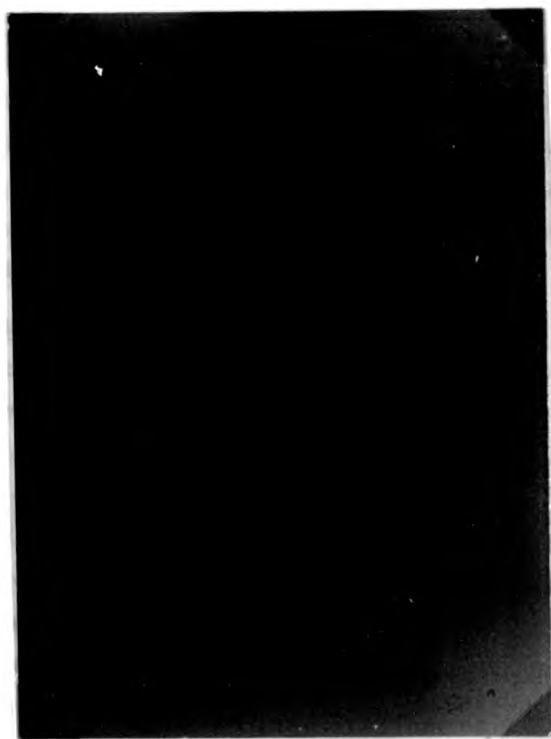
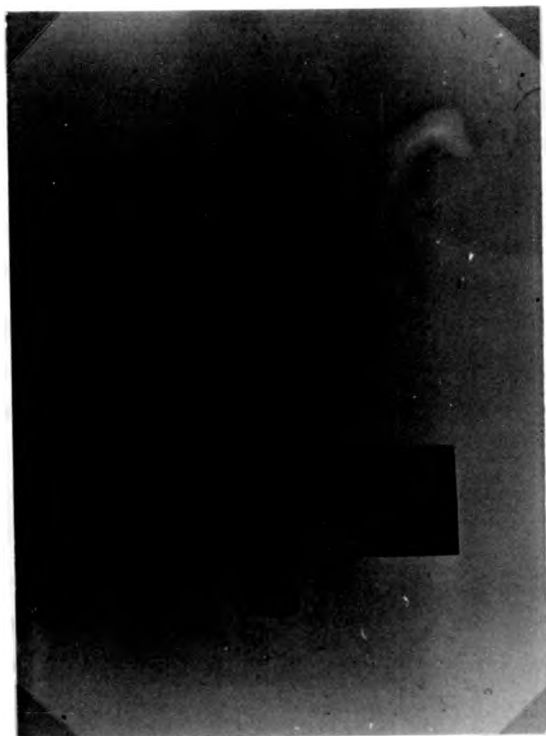


Plate 4

Isoelectric focussing on polyacrylamide gel of  
B. stearothermophilus PNPase according to O' Farrell





### CHAPTER THREE

#### Introduction .

Having purified B. stearotherophilus PNPase to homogeneity, we embarked on a preliminary investigation of the gross structural aspects of the enzyme, such as molecular weight, tertiary structure and amino acid composition. The criteria of homogeneity used were polyacrylamide gel electrophoresis in both denaturing and nondenaturing conditions, and isoelectric focussing. The molecular weight of the native enzyme was then assessed by gel exclusion chromatography and density gradient centrifugation, and the subunit structure investigated by polyacrylamide gel electrophoresis. Antisera raised against the enzyme was cross reacted with E. coli PNPase to assess the similarities of structure between the thermophilic and mesophilic enzymes.

#### Materials and methods.

Discontinuous gel electrophoresis was carried out according to Weber and Osbourne,<sup>158</sup> Buffer systems used were 50 mM sodium acetate, 15 mM sodium borate pH 8.0, Tris-glycine<sup>159</sup> and phosphate.

Gels of between 5 and 7.5 per cent acrylamide were prepared using TEMED rather than riboflavine, with bromopenol blue as tracking dye.<sup>160</sup> Gels were stained in Coomassie brilliant blue r 250 and photography was kindly carried out by Dr. D. W. Hutchinson. Activity stains were carried out as described in chapter 2.<sup>140</sup> Denaturing gels were run in 0.2% SDS 1 mM mercaptoethanol using phosphate or Tris-borate buffer systems. Samples were boiled in 1% SDS 1mM mercaptoethanol immediately before electrophoresis

#### Synthesis of dimethyl suberimidate.

Dimethyl suberimidate was synthesised by the method of Davies and Stark.<sup>161</sup>

Suberonitrile (0.5 g) was dissolved in an ice cold mixture of 2 ml

of methanol and 15 ml of anhydrous ether. Anhydrous HCl was bubbled through the solution for half an hour, and the reaction mixture then left for 24 hours at 4°C. The product was then precipitated by the addition of 10 ml of dry ether, which was then washed with an anhydrous mixture of methanol:ether 1:3. The product was then stored under anhydrous conditions, and used without further purification. No change in crosslinking activity could be detected after one year. The melting point of the product was 211°C (three degrees less than observed by Davies and Stark), and the main product determined to be dimethyl suberimidate by PMR in a Perkin Elmer R 12 60 MHz spectrometer.

#### Cross linking of proteins.

Cross linking experiments were carried out at room temperature for three to twelve hours at pH 8 using concentrations of protein from 1 to 5 mg/ml and 0.1 to 4 mg/ml of dimethyl suberimidate. Control experiments using lactate dehydrogenase (E.C. 1.1.1.27.) were carried out to confirm our ability to successfully operate this technique.

#### N-terminal analysis.

The amino-terminal analysis described by Weiner et al.<sup>162</sup> was used. A stock solution of dansyl chloride (10% w/v in acetone) was stored at 4°C in the dark. Stock solution was diluted 20 times in acetone to give a small amount of working solution at 5 mg/ml.

Purified lyophilised protein (50 - 200 µg) was taken up in 0.1 ml of 0.5 M phosphate buffer pH 8.3, 0.5% SDS. To this material, 0.05 ml of dansyl chloride working solution was added, and the mixture incubated at 100°C for four minutes in a stoppered tube. The mixture was cooled, made 1 mM in mercaptoethanol, and further boiled for a minute. The cooled reaction mixture was

then passed down a Sephadex G.25 filled Pasteur pipette, to remove most of the dansylic acid. Material excluded from the void volume of the column, detected by visual inspection on irradiation of fractions with UV light, was placed in a hydrolysis tube, and the solution made 6 N in HCl. The tube was sealed and heated to 105°C overnight. After cooling, the tubes were opened and the hydrolysates dried down under vacuum.

Ascending chromatography of the hydrolysates was carried out in three different solvent systems, using 15 cm square polyamide plates. The dansylated hydrolysate was dissolved in a minimal volume of 50% aqueous pyridine, and applied in a spot no bigger than 2.5 mm in diameter to the plate by repeated application with a fine capillary, drying between applications with a hair dryer.

A mixture of commercially obtained L-amino acids in equimolar concentration was made up, and dansylated in phosphate buffer to act as standards for comparative purposes. The patterns of spots when separated by the various chromatography systems was essentially as described by Weiner. The chromatography systems used were

- 1) 1.5% formic acid in water,
- 2) Benzene/acetic acid 9:1 v/v,
- 3) Ethyl acetate/acetic acid/methanol 20:1:1 v/v.

#### Amino acid analyses.

Amino acid analyses were carried out by Mr. N. Crabtree of the Sittingbourne Research Centre on a Beckman 120C amino acid analyser, or with the help of Dr. I. Kennedy on a Calbiochem BC 500. Samples were hydrolysed at 105°C in 6N HCl for periods of from 24 to 72 hours. Cysteine content was assessed by performic acid oxidation according



to Hirs,<sup>163</sup> and the content of Serine Tyrosine and Threonine was estimated by extrapolation to zero hydrolysis time. Quantitation of samples analysed by the Calbiochem machine was made by weighing photostat copies of the amino acid peaks, and converting them to nmol equivalents by comparison with peak areas of an amino acid reference mixture, containing 60 nmols of each amino acid.

#### Preparation of antisera.

Antisera was raised by the immunisation schedule of Andrews and Roberts.<sup>164</sup> Antigen (1 mg/ml) in Freund's complete adjuvant was injected at the four ankles and in the neck of rabbits, (0.2 ml injection). After six weeks, antigen (500  $\mu$ g s) was injected into the marginal vein. After eight days, the rabbit was bled from the ear, taking about 40 ml of blood at a time which was allowed to clot at room temperature for three hours. The clot was ringed and the supernatant spun in a bench centrifuge to remove remaining blood cells. The supernatant was then stored in 1 ml aliquots at  $-20^{\circ}\text{C}$ , or further purified by salt precipitation before storage.<sup>165</sup> The crude IgG fraction was titrated against PNPsases by serial two-fold dilution. Admixtures were incubated in normal phosphorolysis buffer containing no polymer for fifteen minutes at  $37^{\circ}\text{C}$ . The sample was spun down in a bench centrifuge and the supernatant assayed for enzymic activity. Double agar diffusion plates were run according to Kabat and Myers.<sup>166</sup>

#### Isoelectric focussing.

The method used was essentially that described by O'Farrell<sup>157</sup> for the first dimension of a two dimensional gel, using LKB ampholines (pH 3 - 10) and slightly larger tubes (10 x 1 cm). Focussing was carried out on duplicate gels, one of which was

analysed for protein after the removal of ampholines by diffusion in a warm (50°C) solution of 1% SDS, and the other sliced for the determination of the pH gradient. 2 mm slices were macerated in 1 ml of water in a scintillation vial, and pH measured by a Pye pH meter.

Molecular weight estimation by gel exclusion chromatography.

Molecular weights were calculated according to Andrews<sup>167</sup> by plotting  $K_{av}$  values of known molecular weight markers against the logarithm of molecular weight. Diagram 3.1 presents the calculated  $K_{av}$ s for the markers described. A column of LKB Aca 34 ultro gel (80 x 2.5 cm) was equilibrated with 50 mM Tris-HCl pH 8.2, 1 mM DTT, 1 mM EDTA under an operating pressure of 15 cm of water. Samples were applied in a minimal volume and elution profiles measured with a uvicord monitor. Both E. coli and B. stearothermophilus PNPase eluted with a  $K_{av}$  of 0.21 ( $\pm .02$ ) on this column.

Determination of sedimentation behaviour.

Using E. coli PNPase as a marker of known S value (9.0 S x 10<sup>-13</sup> secs)<sup>10</sup> we determined the behaviour of B. stearothermophilus PNPase in 5 - 20% sucrose gradients, made up in Tris-HCl 50 mM pH 8.2, 1 mM DTT, 1 mM EDTA, in an MSE 3 x 23 ml rotor. Samples (0.2 ml or less) were applied separately or as admixtures, and centrifuged at 30,000 rpm at 15°C for periods up to 24 hours. Gradients were fractionated dropwise, sucking material from the bottom of the gradients into about thirty fractions per gradient. Both E. coli and B. stearothermophilus PNPases ran exactly in the same position, although absolute S values were not determined.

### Results and discussion.

B. stearothermophilus PNPase, prepared as described in the previous chapter, had an extinction coefficient of  $E_{280}^{1\%} = 5.68$  and a 260:280 nm absorbance ratio of 1.68. These figures are similar to those reported for E. coli PNPase, ( $E_{280}^{1\%} = 4.4 \pm 0.6$ ). The molecular weight of the active enzyme was first determined by gel exclusion chromatography as described above, and was 210,000 daltons with a possible error of twenty per cent, (figure 3.1). T. aquaticus PNPase, however, eluted in the void volume of all gel exclusion media tested, and thus appears to have a molecular weight of at least 500,000 daltons. Further evidence for the molecular weight estimate of B. stearothermophilus by gel exclusion chromatography came from a comparison of the sedimentation behaviour of the enzyme in sucrose density gradients compared with E. coli PNPase. The two enzymes when run separately or as admixtures (differentiated by their thermal stability) showed identical sedimentation behaviour, (E. coli =  $9.0S \pm 0.25$ ). (figure 3.2). The isoelectric point of the enzyme was found to be  $4.1 \pm 0.2$ , using the focussing system of O'Farrell (figure 3.3), which compares with the reported value of 4.1 for E. coli.

Not only were molecular weights and isoelectric points of the two enzymes similar. Antisera raised against impure preparations of B. stearothermophilus PNPase could be titrated against E. coli PNPase with the eventual removal of all anti-B. stearothermophilus PNPase activity. Unfortunately attempts to demonstrate the cross reaction using double agar diffusion techniques were unsuccessful. However, it seems likely from the absorption effects that the two enzymes share some immunochemical determinants.

FIGURE 3.1

Molecular weight estimate of B. stearothermophilus

PNPase by gel exclusion chromatography.

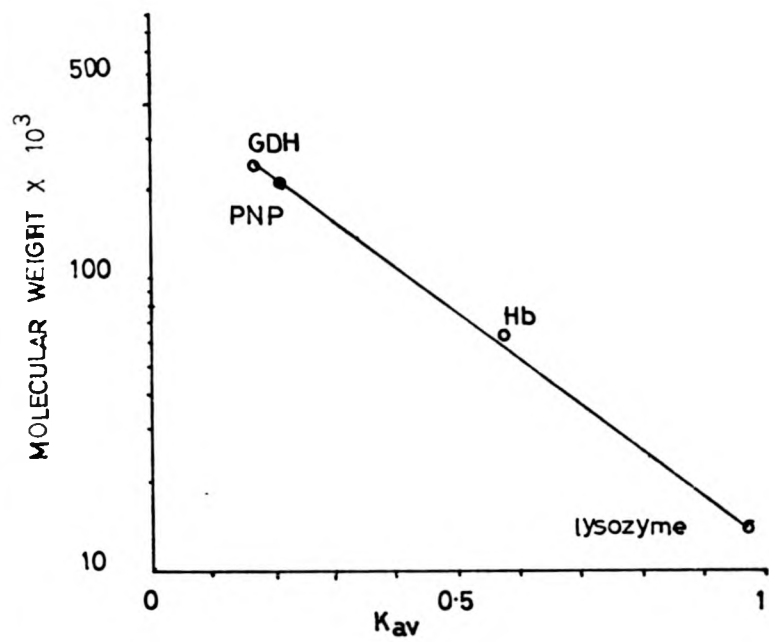


FIGURE 3.2

Comparison of sedimentation behaviour of E. coli  
and B. stearothermophilus PNPases on sucrose  
gradient centrifugation.

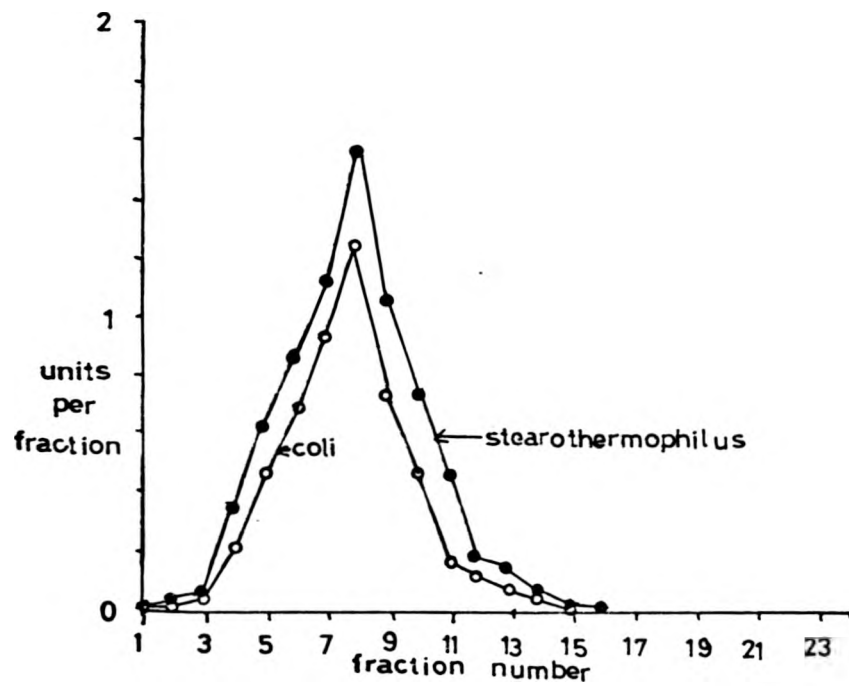
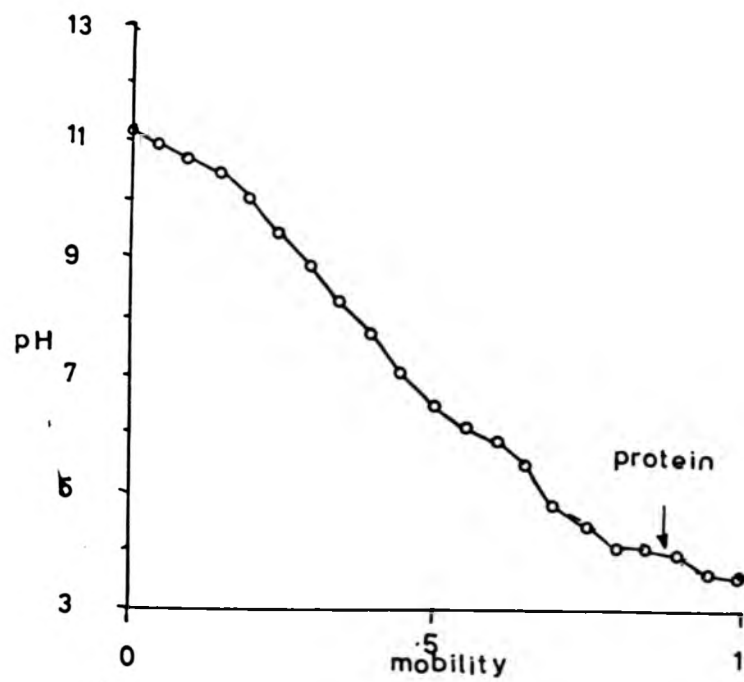


FIGURE 3.3

Isoelectric point of *B. stearrowthermophilus* PNPase.

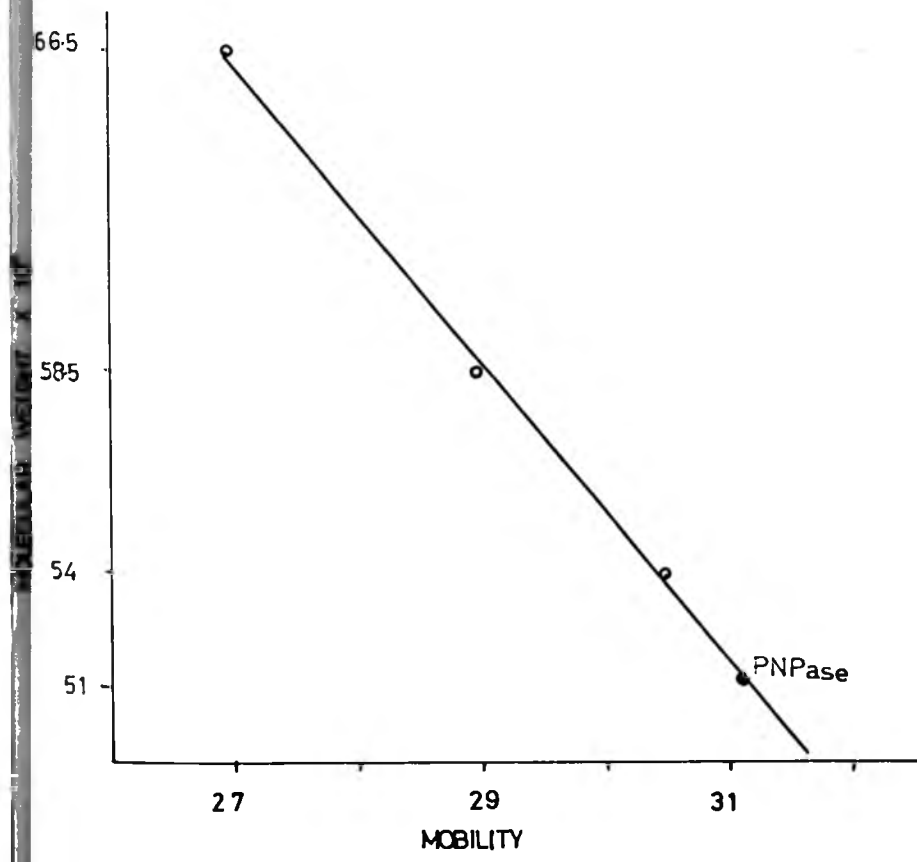


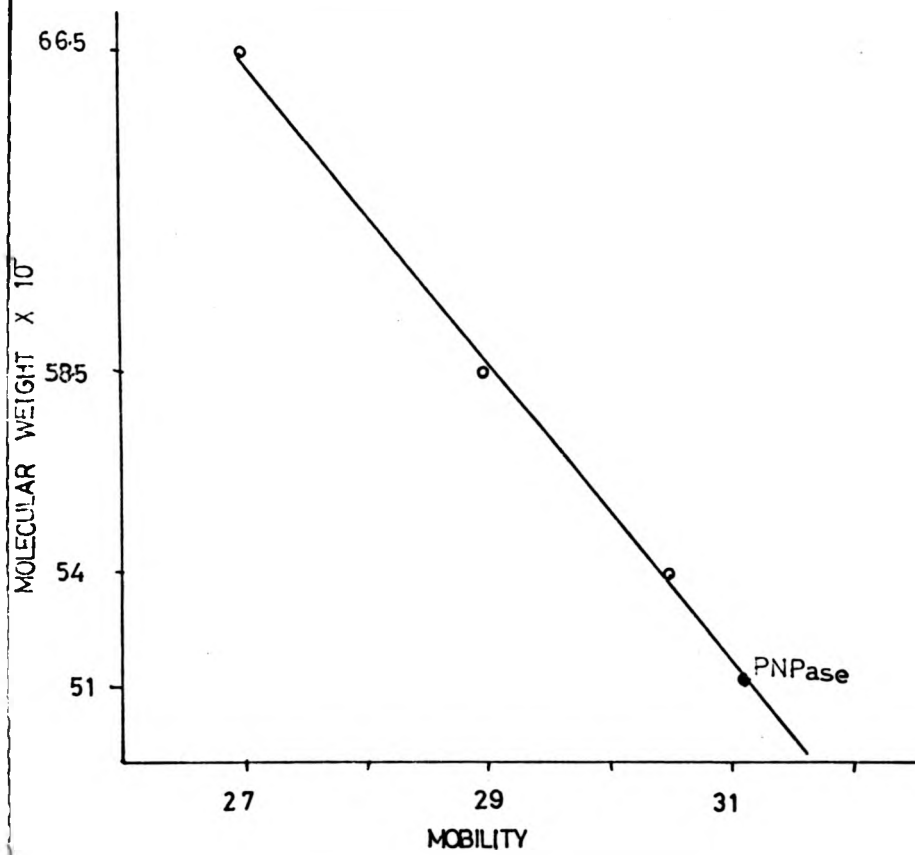


The subunit structures of PNPases have always been subject to dispute, but investigations of the quaternary structure of the B. stearothermophilus enzyme presented an apparently clear picture. On denaturing electrophoresis in SDS, a single band could be distinguished, running slightly ahead of the 58,000 and 54,000 subunits of yeast mitochondrial OS ATPase (E.C. 3.6.1.3). After comparison with these and other markers, a subunit molecular weight of 51,000 daltons was determined, (figure 3.4). Errors of up to 20% in size determination have been revealed, using this technique which gives a range of from 40 to 60,000 daltons.<sup>104</sup> A quaternary structure of four similarly sized subunits thus seemed plausible from this data. N-terminal determinations gave a detectable spot which corresponded to methionine. On chromatography of hydrolysed dansylated B. stearothermophilus PNPase, strong fluorescence on uv irradiation was observed in positions corresponding to dansylic acid,  $\epsilon$ -dansylated lysine and dansylamine. Phenyl-alanine or methionine also showed a much weaker fluorescence. The weak fluorescence corresponding to methionine or phenylalanine was eventually ascribed to methionine after repeated comparisons with standards. Interestingly no fluorescence corresponding to methionine sulphoxide could be detected. Evidence for more than one N-terminal amino acid is thus absent, and it seems likely that the N-terminal is methionine. This provides additional evidence for there being identical subunits in the enzyme. A further method of investigation of quaternary structure was provided by the use of dimethyl suberimidate which cross-links the  $\epsilon$ -amino groups of lysines under the conditions described by Davies and Stark.

FIGURE 3.4

Subunit molecular weight of B. stearothermophilus  
PNPase, determined by denaturing gel  
electrophoresis.





Using this technique with lactate dehydrogenase, we confirmed that the technique was reproducible in our hands. On cross-linking, followed by SDS gel electrophoresis, four major bands could be discerned, at positions corresponding to whole number multiples of the basic subunit molecular weight, (figure 3.5). However, under a variety of conditions, a very strong "dimer" band was apparent whilst "trimer" and "tetramer" bands were very much weaker. Such an effect would be unlikely if the enzyme comprised a symmetrical tetramer of identical subunits. The effect may be the result of the presence of different subunits of similar sizes in the enzyme. Although the predominance of an apparent dimer is puzzling, the presence of four bands is consistent with a tetramer of similarly sized but not necessarily identical subunits. However, the presence of only one band on isoelectric focussing provides further evidence for the presence of a single subunit. Another possible explanation for the anomalous band intensity of suberimidate-linked subunits is an equilibrium between strongly bound dimers and a weaker tetramer. We looked for PNPase activity corresponding to dimeric size by gel filtration at temperatures of up to 70°C at various salt concentrations, but could not detect any activity at such a molecular weight position.

Amino acid analysis data for B. stearrowthermophilus PNPase is shown in Table 3.1. Because of discrepancies in the published data on molecular weights and quaternary structure, the figures are presented as percentage composition figures for each species.<sup>101,104</sup> The similarities in gross composition are noteworthy, and there is no significant difference between relative amounts of polar and hydrophobic residues between B. stearrowthermophilus PNPase and the mesophylic species. This provides further support for structural similarities between the PNPases investigated.

TABLE 3.2

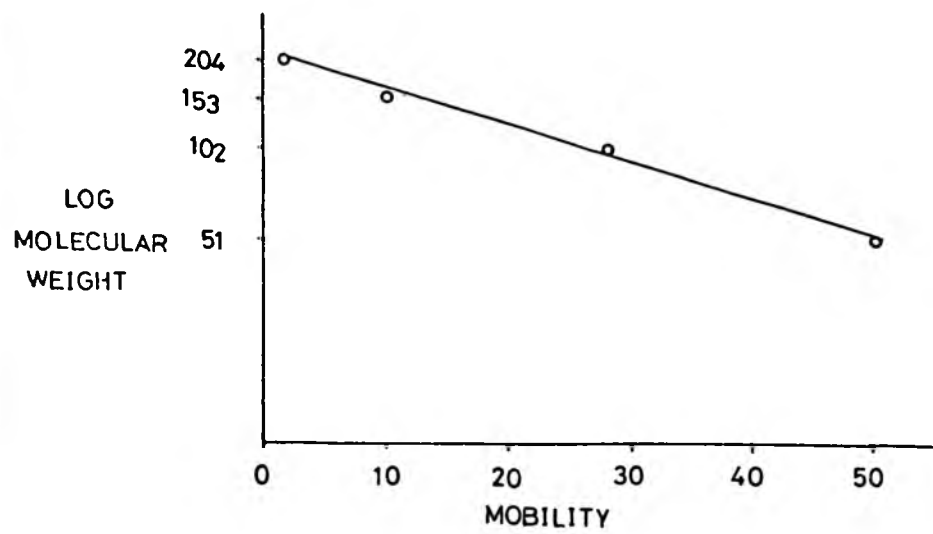
Percentage by weight of amino acids in polynucleotide phosphorylases

Amino Acid	E. Coli PNPase	M. Luteus PNPase	B. Stearothermophilus PNPase
Aspartic Acid	9.48	7.43	10.08
Threonine (b)	5.34	5.21	5.07
Serine (b)	3.86	5.22	3.62
Glutamic Acid	15.42	11.10	11.92
Proline	4.03	4.80	6.86
Glycine	5.18	8.00	7.68
Alanine	6.34	12.40	5.98
Cysteine (a)	0.78	1.02	0.80
Valine	8.67	9.33	9.47
Methionine	2.84	1.63	2.03
Isoleucine	7.19	6.55	5.97
Leucine	7.28	9.00	9.42
Tyrosine (b)	2.79	1.85	3.17
Phenylalanine	4.11	3.23	4.37
Histidine	2.29	1.04	6.64
Lysine	5.91	3.80	3.16
Arginine	7.42	7.17	5.39
Tryptophan	ND	1.03	ND

FIGURE 3.5

Quaternary structure of B. stearrowthermophilus  
PNPase, as determined by denaturing gel  
electrophoresis after suberimidate cross-linking.





## CHAPTER FOUR

### Introduction

The basic parameters of catalytic activity are described for the PNases of T. aquaticus and B. stearothermophilus. The pH dependence, effect of ionic strength and the temperature dependence of the reaction were investigated. The substrate specificities of the two enzymes for the polymerisation and phosphorolysis reactions and the metal cofactor requirements of the enzymes are also described. Particular attention was paid to the synthesis of poly(rG), which is difficult to accomplish with E. coli or M. luteus enzymes. In addition, some observations which may be of physiological significance are presented.

### Materials and methods

Assay systems for polymerisation and phosphorolysis were those described in chapter one. Oligo(rA) and oligo(rG) were prepared by alkaline hydrolysis of polymer followed by alkaline phosphatase treatment as described in reference <sup>141</sup>.

The pH dependence of the reactions was investigated using glycine-NaOH, Tris-HCl and citrate-NaOH buffers for the pH range 9 to 12, 7 to 9 and 5 to 6.8 respectively. Temperature dependence studies were carried out in Tris-HCl buffered to optimal pH at the required temperature.

Binding constants and half activation constants were determined by double reciprocal plots <sup>168</sup> or by the direct linear plot of Cornish-Bowden. <sup>169</sup> Nucleotide substrate concentrations were determined spectrophotometrically from published extinction coefficients. Synthesised polymers were deproteinised by extraction with isoamyl alcohol/chloroform, (5:2 v/v), then dialysed in treated Visking <sup>170</sup>

FIGURE 4.1

The pH optima of B. stearrowthermophilus PNPase.

Phosphorolysis = ●

Polymerisation = ○

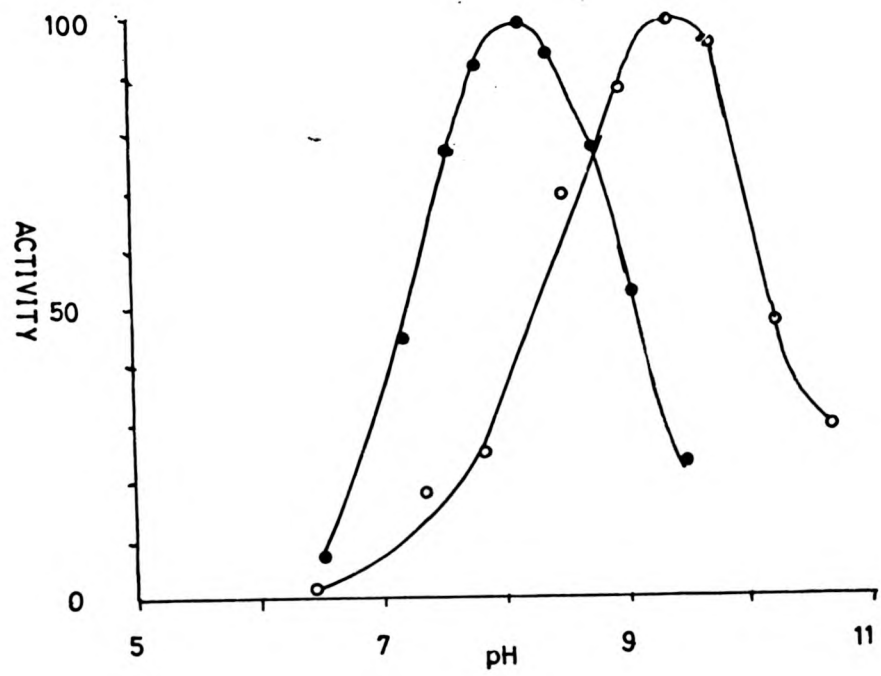
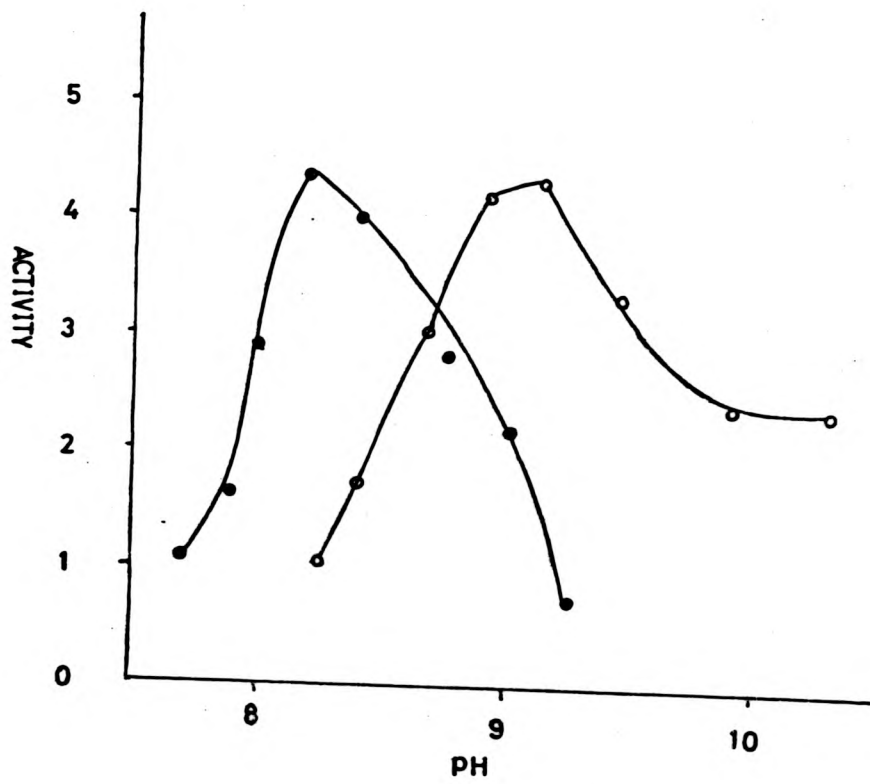


FIGURE 4.2

The pH optima of T. aquaticus PNase.

Phosphorolysis = ●

Polymerisation = ○



tubing before lyophilisation. Molecular sizes were analysed by electrophoresis on polyacrylamide gels in SDS, or on agarose gels (1 - 2%) as described in the appendix. Gels were scanned at the appropriate wavelength in a Gilford spectrophotometer equipped with a gel scanner, or by visual inspection after staining with 0.5 mg/ml ethidium bromide. Approximate molecular sizes were computed by comparison with E. coli rRNA markers or defined size polymers purchased from PL Biochemicals Inc. Molecular sizes of oligomers were also measured by a modification of the method of Tazawa.<sup>171</sup> In the case of polymerising GDP, [<sup>3</sup>H]GDP and [<sup>14</sup>C]UDP of known specific activity were incubated overnight at a variety of molar ratios, with a final substrate concentration of 10 mM. Pancreatic ribonuclease (50 µg/ml) and alkaline phosphatase (200 µg/ml) were then added to the reaction mixture, which was incubated at 37°C for three hours. The mixture was then deproteinised using isoamyl alcohol/chloroform (5:2 v/v) and chromatographed on a G 25 Sephadex column in 0.2 M KCl 0.1% SDS. Material eluting at the void volume was collected, and a sample counted on a Packard liquid scintillation counter to assess the ratio of [<sup>3</sup>H] to [<sup>14</sup>C] and hence obtain an estimate of the mean size of the poly(rG) produced.

#### Results and discussion

In many aspects of catalytic activity the thermostable PNPases showed similar characteristics to mesophylic enzymes.<sup>9</sup> The pH optimum was 9.2 for polymerisation and 8.2 for phosphorolysis, for both enzymes investigated (Figures 4.1 and 4.2). Both magnesium and manganese II were found to be efficient cofactors,

whilst the optimal ratio of metal to nucleotide diphosphates was about 1 to 3 for CDP, UDP and ADP, but 1.1 for GDP. Ionic strength had no significant effect on the rate of reaction of the T. aquaticus and E. coli enzymes, whilst a marked stimulation occurred with pure B. stearothermophilus enzyme (Figure 4.3). Salt stimulation of pure mesophylic PNPases is rarely observed and has been rationalised in terms of accelerating the rate of formation and breakdown of enzyme substrate complexes.<sup>10</sup>

The publication of a report that RNA degradation was slowed by the presence of calcium ions which had been shown to inhibit ribonucleases II and III led to an investigation of the effect of calcium on PNPases.<sup>172</sup> An inhibition of both phosphorolysis and polymerisation was indeed observed at millimolar calcium concentrations, but at lower concentrations a selective activation of the polymerisation reaction was observed, whilst there was no effect on phosphorolysis. This effect is specific for B. stearothermophilus PNPase and could not be observed with pure E. coli or partially purified T. aquaticus PNPases (Figure 4.4). The effect was not a function of substrate or cofactor concentration. By analogy with the micromolar levels of intracellular calcium in E. coli cells this effect may be of some physiological significance. A stabilising effect of calcium ions on thermolysin<sup>173</sup> has been reported and there is some evidence to suggest a general function of calcium ions in the mechanism of thermostability.<sup>174</sup> Nevertheless this does not help to explain the selective activation or the enhancement of catalytic activity described here. It may be that the stereochemistry of a  $\text{Ca}^{++}\text{ADP}$  complex could activate polymerisation in an analogous fashion to that observed by Ikehara with the



FIGURE 4.3

Salt stimulation of B. stearothermophilus PNPase;  
effect of potassium chloride.

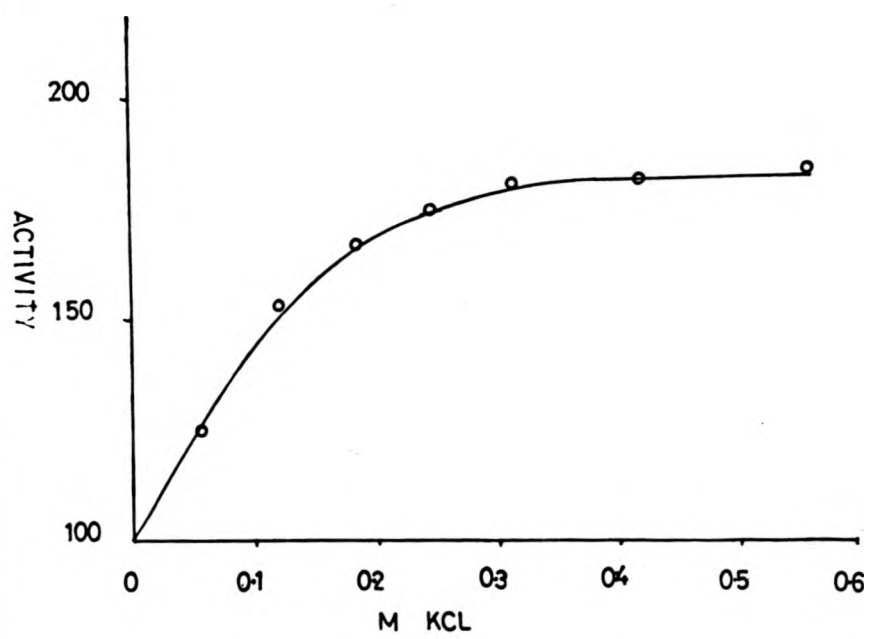


FIGURE 4.4

Effect of calcium ions on the polymerisation of  
ADP by B. stearothermophilus PNPase.

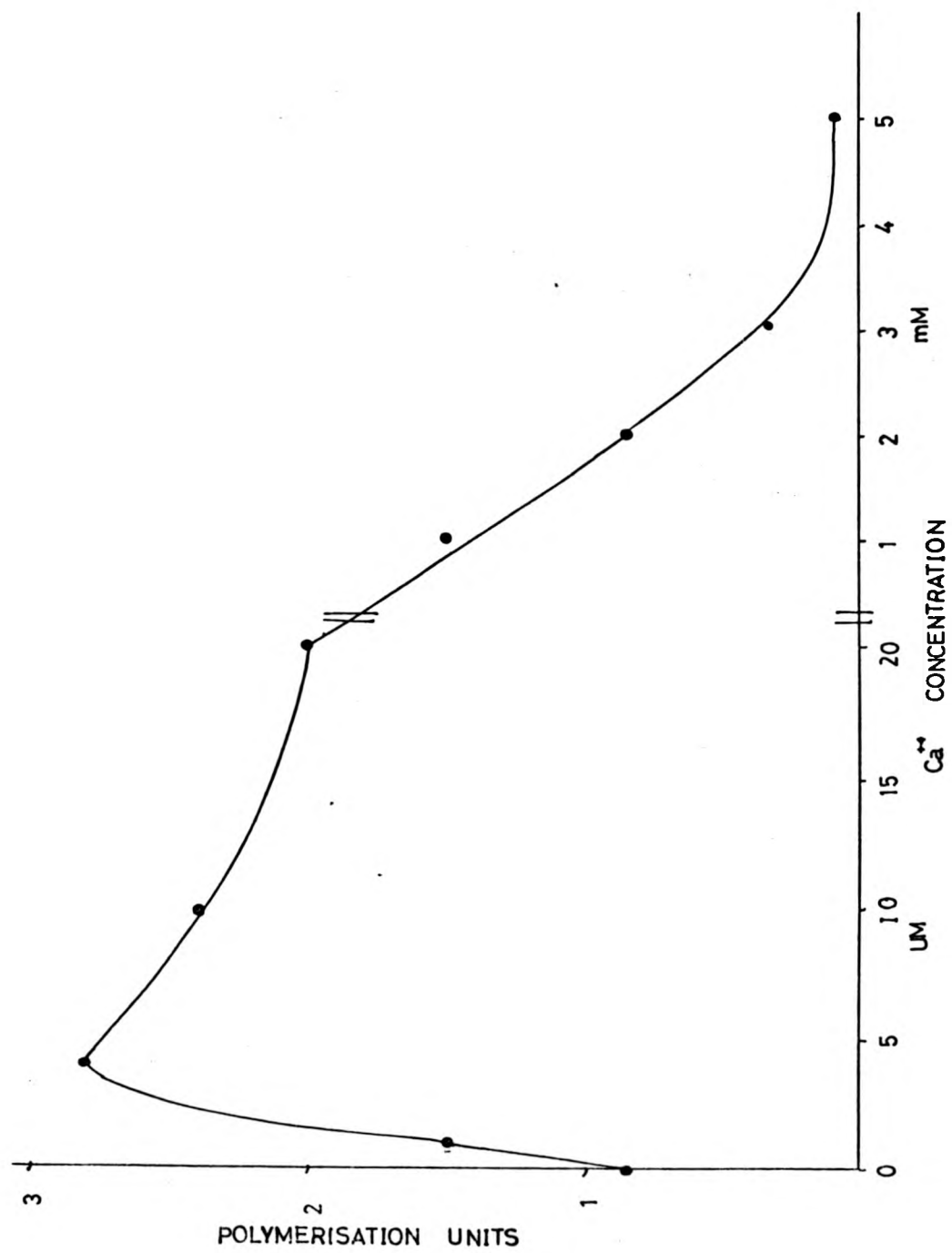


FIGURE 4.5

Substrate specificity of B. stearrowthermophilus

PNPase - phosphorolysis.

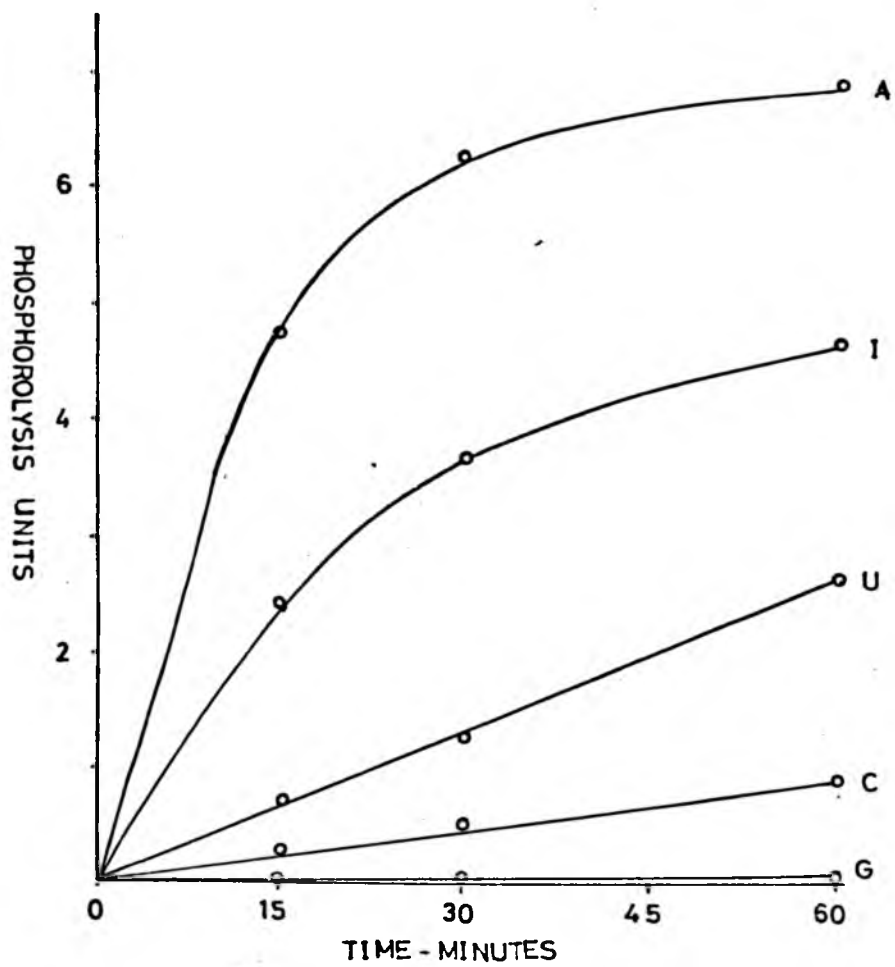


FIGURE 4.6

Substrate specificity of T. aquaticus PNPase

- phosphorolysis.

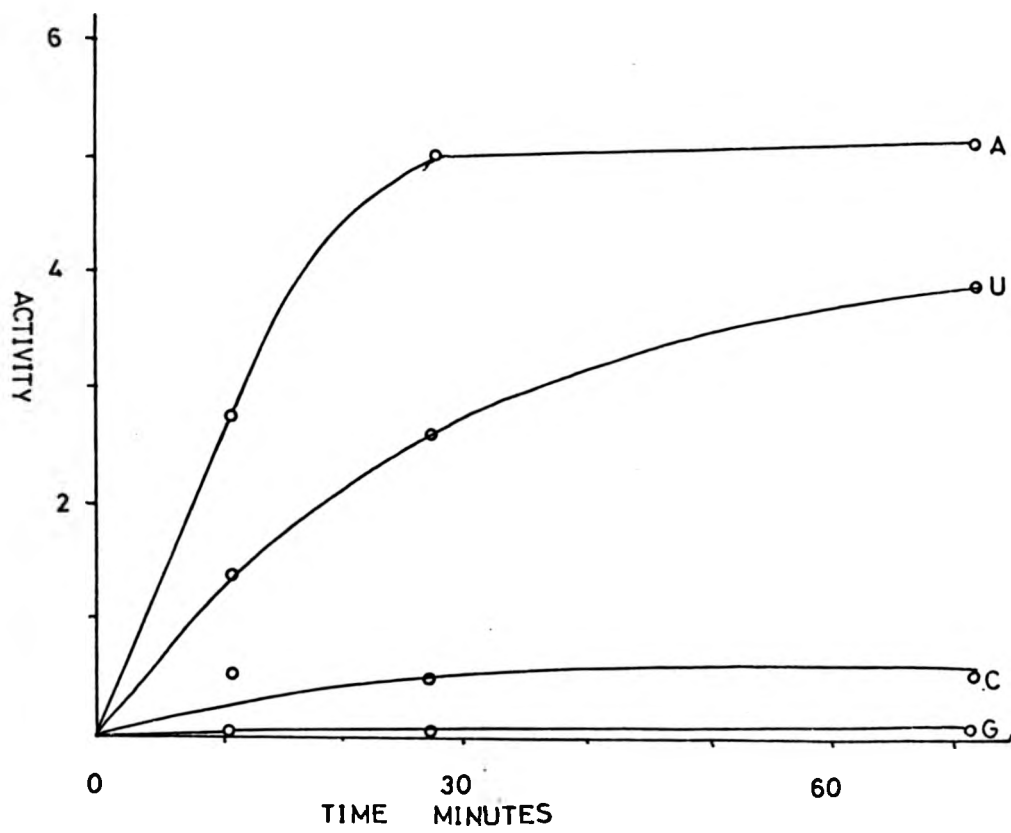




FIGURE 4.7

GDP polymerisation by B. stearrowtherophilus  
PNPase. The effect of spermine, and oligo(rA)  
primer.

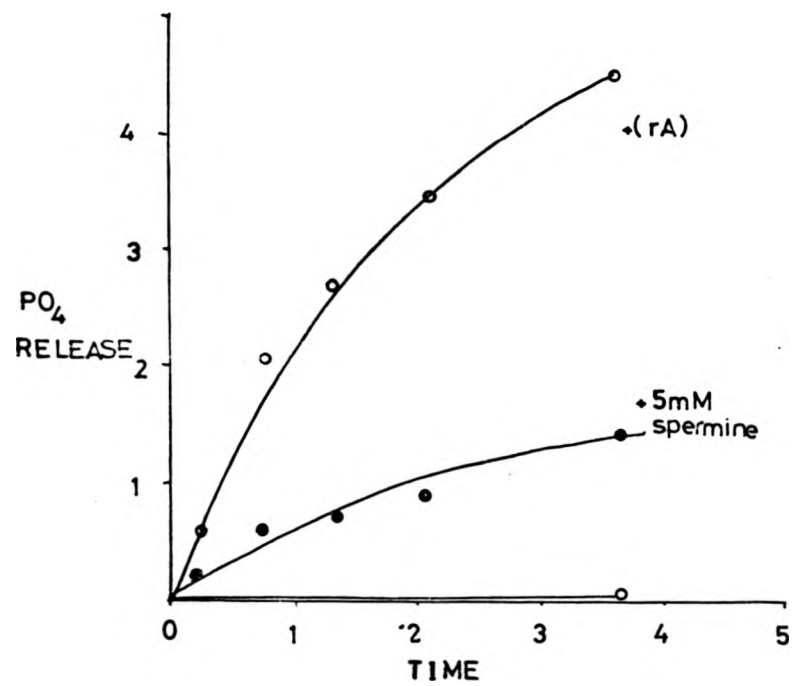


Table 4.1

<u>Compound</u>	<u>Effect on B. stearothermophilus PNPase</u>
Glucose-6-phosphate	0
Fructose-1-6-diphosphate	0
Glucose-1-phosphate	0
Glyceraldehyde-3-phosphate	0
Phosphoribosyl pyrophosphate	0
Phosphoenol pyruvate	0
AMP	± 10% activity
ATP	± 10% activity
Ca <sup>++</sup>	+ 200% polymerisation activity.

Table 4.2

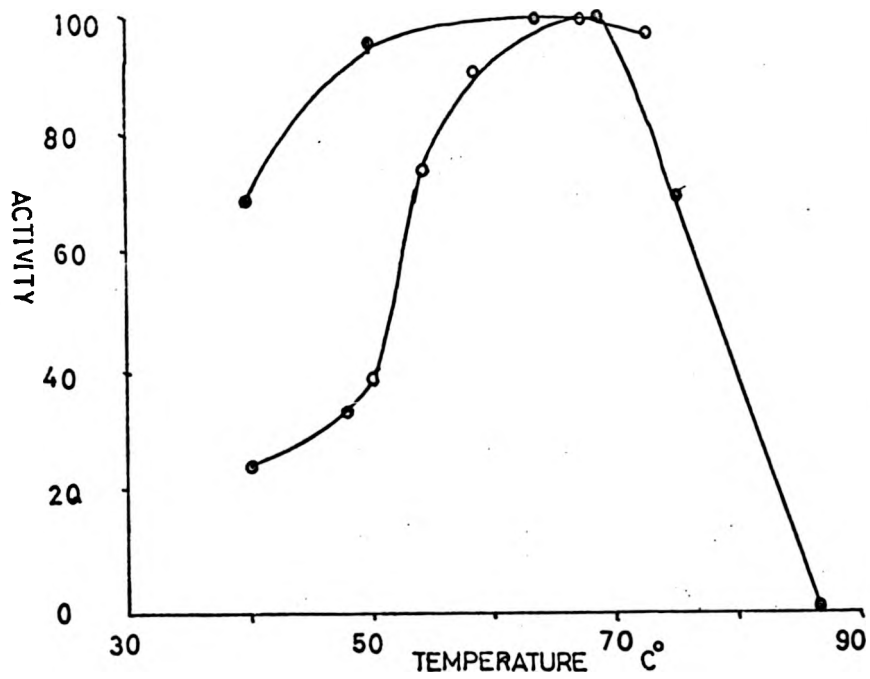
<u>Compound</u>	<u>Km(mM)</u>	<u>V<sub>max</sub>(%)</u>
ADP	2.5	100
UDP	2.9	30
CDP	2.9	27.5
GDP*	2.6	11

\* Using Mn<sup>++</sup> as cofactor.

Oligo(rA)  $\frac{K_a}{0.03 \text{ mM}}$

Figure 4.8

Temperature dependence of phosphorolysis  
by B. stearothermophilus

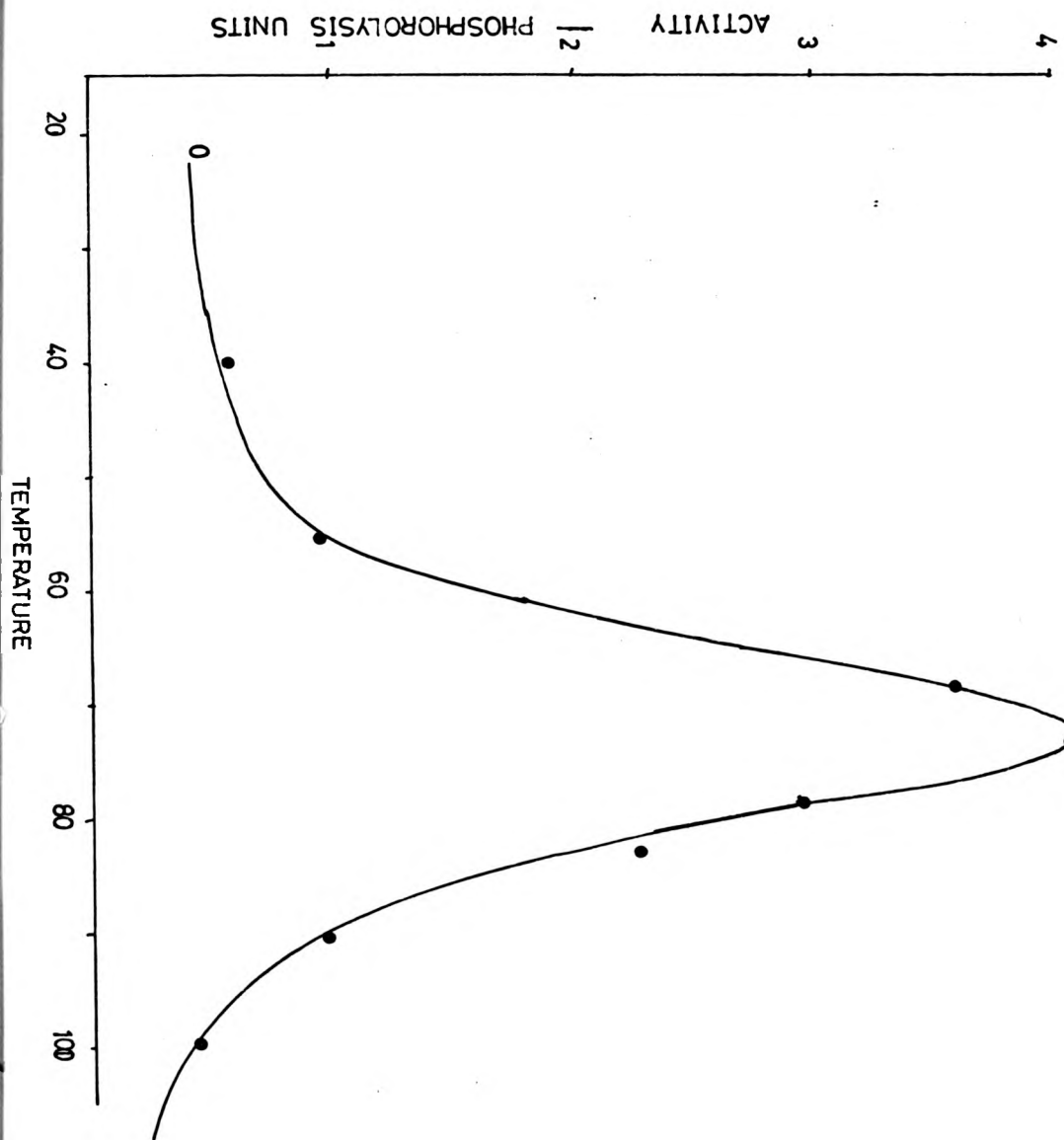


● = assay at 60°C after 30' at temp. shown.

Figure 4.9

Temperature dependence of phosphorolysis

by T. aquaticus



conformationally restricted nucleotide AsDP (Figure 9).<sup>175</sup> This effect is also only detectable at low ratios of the nucleotide to other more suitable substrates.

In the hope of elucidating the role of PNPase in vivo, a number of molecules of physiological significance were incubated with the enzyme to assess any possible regulatory effects. Neither variation of the energy charge by the addition of various ratios of ADP, ATP and AMP to the reaction mixture,<sup>176</sup> nor any of the small molecules investigated, had any profound effect on the enzyme activity, (Table 4.1).

The relative affinity of the two enzymes for various naturally occurring substrates are present in Figures 4.5 and 4.6 and Table 4.2. The  $K_a$  for priming by oligo(rA) of approximately thirty residues in length is also shown. The variation of substrate specificity through the purification of B. stearothermophilus PNPase has been remarked upon in the second chapter. Initial studies suggested that the enzyme would be highly efficient in polymerising GDP, in comparison to mesophylic enzymes. It can be seen from Figure 4.7 that this is not so. Incubation of the enzyme with spermine showed an enhancement of unprimed activity which was not observed in the presence of oligo(rA). This suggests that spermine in some way acts to relieve primer dependency, perhaps by neutralising charges on the oligomeric initial product of polymerisation and allowing easier release from the active site.

Using oligo(rG) as a primer, we could observe no stimulation over unprimed activity with B. stearothermophilus PNPase. If the sole requirement for a primer is to present a free 3' hydroxy group at the active site, one would expect a biphasic reaction plot with the ratio of the two slopes being the same as the ratio of the



V maxs of the two substrates. This effect is not observed. Kapuler<sup>71</sup> has shown with the PNPase from E. coli that neither p8rGpA nor pAp8rG function are effective primers, implying that a specific conformational arrangement of the primer is necessary for activity. Oligo(rG) is presumably not capable of assuming such a conformation. This sheds a puzzling light on the fact that GDP can be polymerised, as one would expect that after the primer of oligo(rA) had been sufficiently elongated, poly(rG) residues would occupy the priming site and presumably inhibit the reaction. This does not happen (Figure 4.7). There remains the possibility that primer molecules are not incorporated into the subsequently synthesised polymers, but act in some other way to enhance polymerisation. The early literature demonstrating the incorporation of radioactive oligomers into polymer is not conclusive.<sup>78,79</sup>

A report by Thang<sup>53</sup> that E. coli PNPase copolymerised GDP and other nucleotide diphosphates to give polymers of composition which did not reflect the initial molar proportions of substrates led to an investigation of this phenomenon with B. stearothermophilus PNPase. As can be seen from diagram 4.2, the polymer composition roughly reflects the initial substrate composition of the incubation. Nevertheless, as also noted by Thang, the proportion of GDP molecules incorporated into polymer seems to always be slightly higher than the initial ratio of substrates. Having prepared the double labelled copolymers, degradation with ribonuclease as described in methods was used to prepare approximately sized poly(rG) molecules with a terminal U residue. This simple approach to sizing molecules may be of general use for the preparation of polymers of which the terminal residue is of no great importance. The size dependency of poly(rI)(rC) for interferon induction is a good example.<sup>177</sup>

TABLE 4.2

Copolymerisation of [ $^3\text{H}$ ]-GDP and [ $^{14}\text{C}$ ]-UDP

GDP/UDP* <u>Input Ratio</u>	(G:U) Base Ratio of High Molecular Weight <u>Product</u>
10	18
20	19
30	38
40	65

\* Specific radioactivities [ $^3\text{H}$ ]-GDP 24,000 cpm/mg, [ $^{14}\text{C}$ ]-UDP 26,000 cpm/mg. Reactions were carried out overnight under polymerisation assay conditions with final substrate concentrations of 10 mM.

Pancreatic RNase (50  $\mu\text{g}/\text{ml}$ ) and alkaline phosphatase (200  $\mu\text{g}/\text{ml}$ ) were then added and the mixture incubated at 37 $^{\circ}$  for 3 hours. Deproteinisation with isoamyl alcohol: chloroform (5:2 v/v) followed by chromatography of the aqueous layer on Sephadex G 25 with elution by 0.1% SDS and 0.2M KCl solution gave the products in the void volume.

The temperature dependence of the phosphorolysis reaction for B. stearothermophilus PNPase is detailed in Figure (4.8), where a very rapid fall off in activity at temperatures above 70°C can be seen. Impure T. aquaticus PNPase preparations were stable at up to 85°C. However, the partially purified enzyme showed a similar temperature optimum to B. stearothermophilus PNPase. The characteristic lack of activity of enzymes from obligate thermophiles at temperatures below about 50°C can be seen in Figure (4.9).

## CHAPTER FIVE

### Introduction.

Having investigated the catalytic activity of B. stearothermophilus and T. aquaticus PNPases with naturally occurring substrates, a number of modified nucleotide diphosphate were synthesised and tested as substrates for the enzymes. Of the purines investigated, 8brADP and 8brGDP were chosen because of their conformational restriction to the syn form at room temperature,<sup>63,64</sup> whilst 8azGDP had the extra advantage of being a potentially useful photo-affinity probe. Of the purines tested, 5ohCDP has shown significant anti-viral activity. 5nh<sub>2</sub>UDP was also investigated. Following the report that deoxy substrates could be effectively utilised by PNPases under certain conditions, the phosphorolysis and polymerisation of various deoxynucleotide substrates were investigated. The possibility of direct DNA sequencing by the stepwise degradation of DNA molecules whereby [<sup>32</sup><sub>p</sub>] of very high specific activity may be incorporated into the products of the degradation is an attractive prospect.

### Materials and methods.

Purified T4 DNA was the gift of Dr. Avery. Dimethyl phosphonate was the gift of Dr. D.W. Hutchinson. 8azADP was the gift of I.L. Cartwright and 5amUDP of N. Obertelli.

### Synthesis of 8brGDP.

GDP (0.5 mmole) in formamide (4 ml) at 0°C was treated with Br<sub>2</sub> (1 mmole) in chloroform (1 ml) and the mixture stirred for one hour. The solution was then ether extracted (2 x 20 ml) and the aqueous layer treated with ethanol:ether (1:1 v/v).<sup>51</sup> The precipitated 8brGDP was purified on an A 25 -Sephadex column, eluted with a gradient of 0 - 0.2 M triethylammonium bicarbonate.

#### Synthesis of 8azADP .

8 azido adenosine diphosphate was the kind gift of I. L. Cartwright. The material was synthesised by bromination of AMP according to Ikehara and Uesugi<sup>123</sup> to give 8brAMP, which was purified by DEAE Sephadex A-25 chromatography. 8azAMP was then made by heating at 70°C for two days a mixture of 8brAMP (1 g) tetramethyl guanidinium azide (1.26 g) in dimethyl formamide (50 ml). Dimethyl formamide was then distilled off under vacuum, the residue extracted several times with ether to remove unreacted azide, and the material purified on an A-25 Sephadex column with a linear gradient of 0.05 - 0.15 M lithium chloride. The purified material was then converted to the triethylammonium salt by block elution from another A-25 DEAE Sephadex column with 0.15 M triethylammonium bicarbonate. The 8azAMP was then converted to the diphosphate by the method of Moffatt and Khorana,<sup>66</sup> and finally purified on an A-25 Sephadex column using a gradient of 0-0.2 M triethylammonium bicarbonate.

#### Synthesis of 8brADP .

Although direct bromination of ADP is possible, better yields are obtained by bromination of AMP followed by conversion to the diphosphate via the phosphomorpholybdate. Synthesis of 8brADP was carried out by bromination followed by phosphorylation as described above.

#### Synthesis of 5ohCDP .

The method of Eaton and Hutchinson<sup>132</sup> was used for the synthesis, involving the treatment of CDP with Bromine in aqueous collidine. CDP (trisodium salt 300 mg) was dissolved in 3 ml of water at 0°C

to which  $\text{Br}_2$  was slowly added, until a yellow colour persisted. A little cyclohexene was then added to remove excess bromine, followed by 1.5 ml of 2,4,6-collidine. The mixture was then incubated at  $37^\circ\text{C}$  for two hours, then ether extracted (4 x 4 ml), and the aqueous layer applied to a TEAE-cellulose column ( $\text{HCO}_3$  form). The column was eluted with a linear gradient of tri-ethyl-ammonium bicarbonate, 5ohCDP eluting at about 0.1 M. The pooled fractions containing 5ohCDP were evaporated to dryness and excess bicarbonate removed by repeated evaporation after addition of methanol.

#### Polymerisation techniques .

Initial studies with the modified bases were carried out at  $70^\circ\text{C}$  with manganese II as cofactor. Variation of temperature, the use of magnesium as cofactor, and the addition of spermine and formamide were investigated. Up to 12% formamide in the polymerisation buffer had no deterrent effect on enzyme activity. As well as the modified nucleotide diphosphates, commercially obtained dADP was investigated as a substrate.

Polymerisation was detected by a phosphate-release assay. Deproteinised reacted samples were also chromatographed on G-25 Sephadex columns in 0.1% SDS 0.2 M KCl to detect any nucleic acid material eluting in the void volume.

#### Phosphorolysis of atypical substrates .

In order to detect low levels of phosphorolysis  $^{32}\text{P}$  of very high specific activity was used in the conventional assay, described in chapter 2. The products of the reaction were also investigated using thin layer chromatography in a solvent system comprising 1-propanol-concentrated ammonium hydroxide water 6:3:1.

The counts co-chromatographing with appropriate nucleotide diphosphate markers, determined by visual inspection on UV irradiation, were assessed and compared with control samples from unincubated reaction mixtures.

### Results and discussion .

The results of attempted polymerisations of modified bases can be seen in Table 5.1. All the modified bases were good competitive inhibitors for B. stearothermophilus PNPase. This disappointing result may be interpreted as a consequence of the more rigidly arranged active site necessary for in vivo function at the operating temperatures of thermophiles. The fact that 5ohCDP is quite easily polymerised by E. coli PNPase and 8azADP<sup>71</sup> can be polymerised to oligonucleotide products, stress the narrower range of substrate specificity of the thermostable enzyme. An early report also suggests that 8azaGDP can be polymerised to oligonucleotides of about ten residues in length.<sup>74</sup>

Many attempts to phosphorolyse T4 DNA, oligo(dT) prepared according to chapter 2, or sheared alkaline phosphatase treated DNA from calf thymus and salmon sperm also met with no success. Irreproducible  $K_i$  values of a high order ( $> 10$  mM) were found for deoxypolynucleotides with poly(rA) as substrate. Up to 0.5% phosphorolysis with calf thymus DNA was observed, but this is within the possible range of contamination by ribopolynucleotides of either enzyme or substrate.

Jordan<sup>178</sup> has discussed the conformation of some of the modified nucleotide diphosphates mentioned here from a theoretical viewpoint. 2' deoxynucleotides exist predominantly in an 'anti' conformation whilst 8brA<sup>179</sup> and 8brG have a very small probability of attaining an anti conformation (with an energy barrier of about 50 Kcal/mol). These calculations are confirmed by X-ray data.<sup>178</sup> That the easing of the transition with higher temperature does not improve the yield of polymer suggests thermostable PNPases will be of little use in the construction of modified polymers. However, this 'rigidity' has



TABLE 5.1

Parameters of inhibition by atypical bases and other  
substrates for B. stearotherophilus PNPase

Compound	Ki mM
8br ADP	0.21
8br GDP	0.14
8az ADP	0.12
Pyrophosphate	0.04
Methylene diphosphonate	0.28
5ohCDP	0.62
5nh <sub>2</sub> UDP	Not determined

simplified our structural studies, and allowed an unambiguous interpretation of the quaternary structure of a PNPase to be made. In this respect B. stearothermophilus PNPase has advantages over PNPases from more conventional sources. However, the metabolic role and catalytic mechanism of PNPases from all sources remains elusive.

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APPENDIX

The Location of SV40 specific sequences in the pre-mRNA  
of transformed cells.

## Contents

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### Introduction

#### a) The transcriptional unit in eukaryotes.

Although the mechanism of information transfer in prokaryotes and eukaryotes is broadly similar, control of gene expression appears to differ significantly between the two.<sup>[1]</sup> In prokaryotes, those mRNAs which have been studied are degraded randomly and quickly with half-lives of a few minutes<sup>[2]</sup>, suggesting that transcriptional controls are of over-riding significance in the control of protein synthesis. However, eukaryotes have mRNAs which are much longer-lived, with half-lives of the order of a cell life time,<sup>[3]</sup> up to several weeks<sup>[4]</sup>, and there is good evidence to suggest that translational controls play an important role in the modulation of their gene expression, even though transcriptional products are specific to cell type.<sup>[5]</sup>

The structural and functional organisation of the genetic material in prokaryotes has been described by Jacob and Monod,<sup>[6]</sup> but whether a similar arrangement is present in eukaryotes is questionable. Little evidence is presently available,<sup>[7]</sup> but most theories of chromatin organisation tacitly assume an operon type arrangement.<sup>[8,9,10]</sup>

The reasons for our comparative ignorance of the mechanism and control of protein synthesis in eukaryotes may be primarily ascribed to the difficulties of genetic analysis of higher organisms, to the large size of their genomes, and to the complexity of their RNA metabolism. One approach to the problem of transcriptional organisation and gene expression in higher organisms is to use DNA viruses covalently incorporated into the host genome as model systems for normal gene expression.<sup>[11]</sup> This offers the advantage that purified viral DNA can be used as a probe for the appearance of virus messenger sequences, and



hybridisation techniques can be used to purify both RNA transcripts and the region of the genome containing the virus sequences. A structural and functional analysis of a purified transcriptional unit may thus be made.

b) SV40 as a model system

SV40 was discovered in 1960, during a survey of endogenous viruses found in monkey cell tissue culture.<sup>[12]</sup> At that time, polio virus vaccine was being raised in monkey cells as a precaution against introducing viruses from human tissue culture cells into the population.<sup>[13]</sup> In 1962, SV40 was shown to be oncogenic in some organisms<sup>[14]</sup>, which led to a great deal of interest in the virus. It has subsequently been shown<sup>[13]</sup> that virus infection may be either productive or abortive, depending on the genetic character of the host cell. Productive infection leads to cell death after about thirty-six hours, during which time the virus enters the cell, uncoats, replicates its DNA and initiates the replication of host cell DNA. Virus capsid protein and other structural proteins are synthesised and, together with DNA, assembled into virus particles released at the time of cell death. Cells which allow the replication of the virus in this fashion are called permissive.

Non-permissive cells show no capsid protein or virus DNA synthesis on infection, and no progeny virus are released. After a few divisions, the cells may return to normal, having transiently shown signs of a transformed phenotype, or in a few cases they may become stably transformed. (Figure 1). Transformation of permissive cells may also occur when they are infected with a defective virus which is incapable of replication. Cell hybridisation between permissive cells and non-permissive cells infected with SV40 leads to the

FIGURE 1

Time course of events after SV40 productive  
infection; from ref. 13.

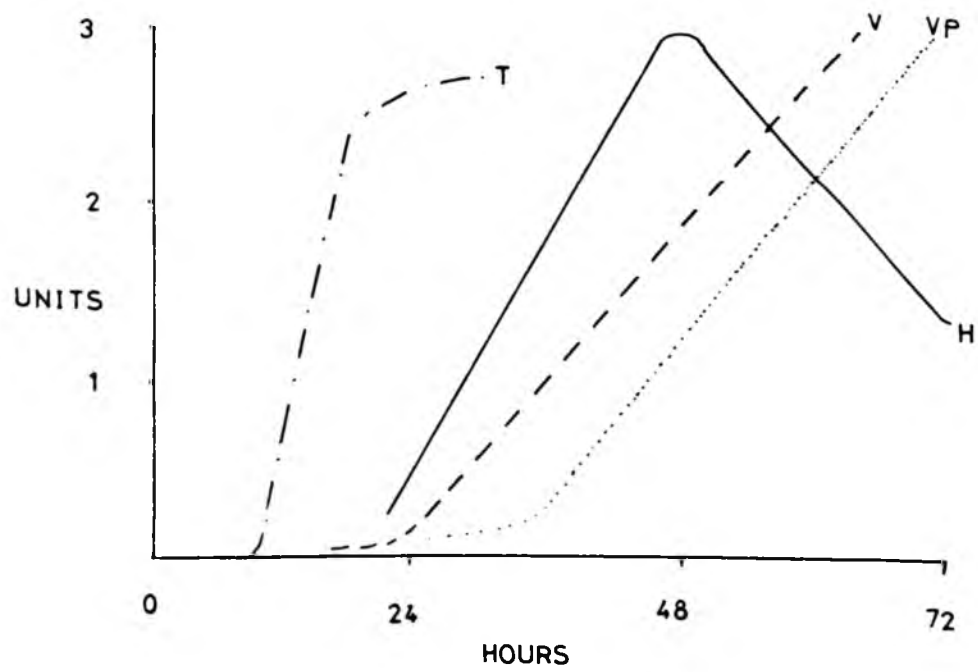
T = T antigen

H = host DNA

V = virus DNA

VP = virus progeny

The ordinate is calibrated in arbitrary units.



appearance of virus, which suggests that loss of a function that permits virus replication is responsible for the non-permissive nature<sup>[15]</sup> of the cells.

SV40 is a member of the papova group of viruses, containing a double stranded closed circular DNA molecule of molecular weight  $3.6 \times 10^6$  daltons. This DNA is a right-handed supercoil, with about fifteen turns less per helix than would be expected in a relaxed circle, and is designated form 1 DNA. Nicked DNA is equally infective, but non-supercoiled, with a sedimentation coefficient of 16S as opposed to 20S for form 1 DNA. Closed circular non-supercoiled DNA is referred to as form 11 DNA. Linear SV40 DNA molecules are designated form 111. In order to denature and separate the DNA strands of SV40, DNA form 1 must be nicked or cleaved to give form 11 or form 111. That SV40 DNA is covalently incorporated into the host genome of transformed cells has been demonstrated by both DNA/RNA<sup>[16]</sup> and DNA/DNA<sup>[17]</sup> hybridisation techniques, using either purified tritiated viral RNA transcribed by *E. coli* RNA polymerase, or radioactive single stranded SV40 DNA. Both methods provide evidence that there are multiple copies of the SV40 DNA in a variety of transformed cells. This fact is corroborated by the phenomenon of virus rescue, where cell hybridisation between permissive and transformed cells may lead to the appearance of infective SV40 particles.<sup>[15]</sup> Between one and nine copies of SV40 per host genome have been observed in different transformed cell lines.<sup>[13]</sup>

The discovery that *E. coli* RNA polymerase asymmetrically and completely transcribes one strand of form 1 DNA<sup>[18]</sup> has allowed a close analysis of transcription in lytic and non-productive infection.

In lytic infection, viral RNA sequences which appear early in infection are not identical to those appearing later.<sup>[19]</sup> About a third of SV40 DNA sequences are found in early RNA, whilst late sequences contain both early and other sequences. Using hybridisation studies with the separated strands of SV40 DNA, it has been shown that early and late RNA sequences are complimentary to different DNA strands, so that a transcriptional strand switching mechanism operates in lytic infection.<sup>[20]</sup> 30 - 40% of one strand codes for early sequences, whilst 60 - 70% of the complimentary strand is transcribed later. That virus specific RNA can be isolated from polysomes<sup>[21]</sup>, and contains long tracts of poly A<sup>[22]</sup> suggests a messenger function for the RNA. Whether SV40 integrates into the host genome in lytic infection is uncertain, but the presence of virus sequences in giant nuclear RNA molecules suggests that it does.<sup>[23]</sup>

In transformed cells, hybridisation studies have demonstrated the presence of virus specific RNA species.<sup>[24]</sup> Many transformed lines contain a larger proportion of early strand RNA sequences than are expressed in lytic infection. Instead of thirty per cent of the early strand being transcribed, up to eighty per cent transcription has been observed, comprising mainly non-informative sequences. A small and variable amount of late strand RNA has also been detected in some transformants. The necessity of early gene expression for the maintenance of transformation has been deduced from these data.<sup>[13]</sup>

The presence of virus specific sequences in giant nuclear RNA molecules has been demonstrated by a number of groups.<sup>[25, 26, 27]</sup> Whereas cytoplasmic sequences sediment at around 20S, 60 to 70S molecules containing virus, and presumably host sequences, have

been found in the nuclei of transformed cells. That some of the viral sequences are informative is suggested by the expression of virus specific antigens in all transformants. The presence of poly A tracts in virus sequences also suggests a messenger role for the RNA. Hence there is considerable evidence that SV40 gene expression in infected cells occurs by similar mechanisms to those involved in endogenous transcription and translation.

c) Nuclear RNA metabolism

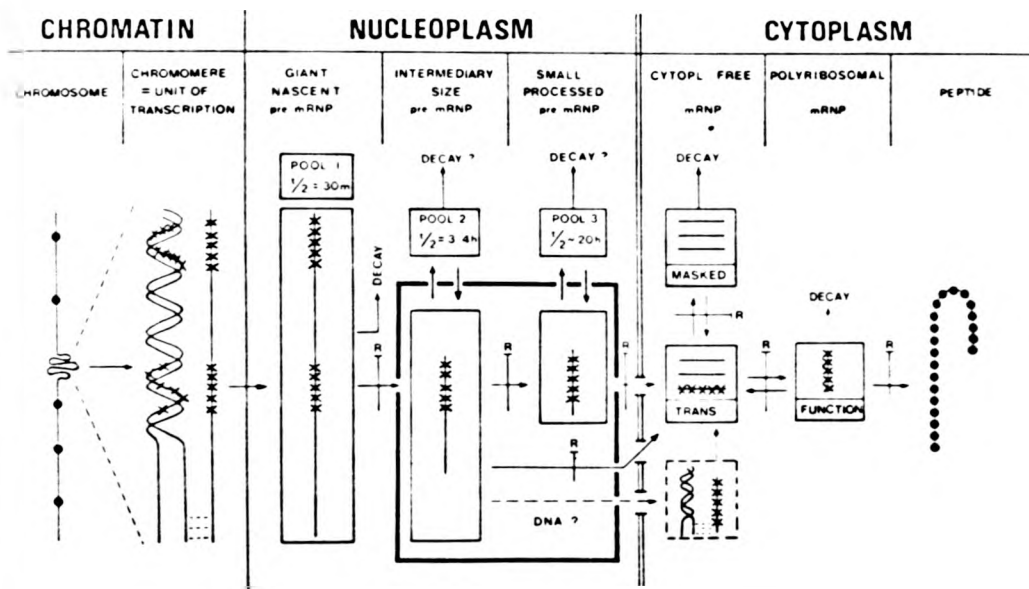
The sequence of events in the synthesis of duck globin is presented in Figure 2, and has recently been reviewed. [28, 29] Briefly, there are three classes of nuclear RNA containing messenger sequences; giant molecules of molecular weight  $5 - 20 \times 10^6$  daltons with half-lives of the order of minutes, intermediate size pre-mRNA of molecular weights  $1 - 5 \times 10^6$  daltons and half-lives of three to seven hours, and small pre-mRNA of a similar size to mRNA with a half-life of more than fifteen hours. The exact relationship between the fractions is very difficult to elucidate by pulse chase experiments, because of the small amount of mRNA produced, the complex and fast metabolism of the large RNA fractions, and the molecular weight range of nuclear RNA species. However, it seems plausible that there is a precursor-product relationship between the three classes and recent work by Darnell [30] using pre-mRNA adenovirus containing sequences as a model, provides evidence of a physical precursor-product relationship between the larger and smaller fractions of pre-mRNA. Polyadenylation occurs at the 3' end of the intermediate sized RNA, and this material may well be the precursor of the poly A sequences found attached to mRNA in the cytoplasm. [31] This not only provides evidence for a precursor product relationship between

FIGURE 2

Information transfer and mRNA synthesis in  
eukaryotes; from ref. 28.

R represents possible regulatory steps.

The black box encloses those steps which  
are at present hypothetical.





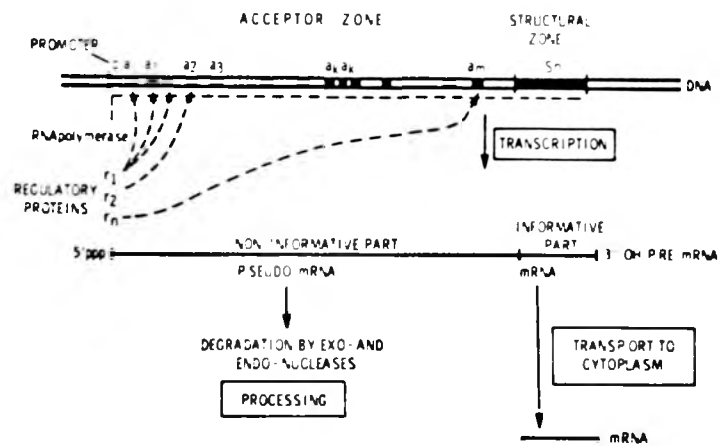
the RNA species involved, but also suggests that messenger sequences are located towards the 3' end of the messenger precursor molecules. Competitive hybridisation experiments also favour this interpretation,<sup>[32, 33]</sup> and a model of chromatin structure has been proposed to take into account these features of mRNA processing, (Figure 3).<sup>[8]</sup> Nevertheless, when carrying out hybridisation experiments on bulk cellular RNA, there is always the possibility of error, as putative messenger sequences are only identified by their hybridisation characteristics. For this reason, work with a defined messenger species such as SV40 is preferable.

d) The localisation of virus specific sequences in pre-mRNA

The work described is a preliminary study on the location of virus specific sequences in giant nuclear RNA molecules of a transformed cell line. Three approaches to this problem have been made using DNA/RNA hybridisation techniques to identify viral sequences. The first approach, carried out by Mantieva and Georgiev<sup>[34]</sup> was to irradiate monolayers of transformed cells with ultraviolet light at 254 nm. With increasing irradiation, progressively smaller nuclear RNA molecules are observed. This is probably due to premature termination of transcription, owing to the formation of pyrimidine dimers. Hence the nuclear RNA should be shortened at its 3' end, with a consequent loss of sequences present there. Preliminary experiments using tritiated pre-mRNA hybridised to SV40 DNA have shown an apparent enrichment of virus sequences with decreasing molecular size, suggesting the absence of virus specific sequences at the 3' end of the molecule. This is a surprising result, as messenger sequences appear to be located towards the 3' end of their precursor molecules. In order to obtain

FIGURE 3

The eukaryotic transcriptional unit, according to Georgiev, (ref. 8).



further information, high molecular weight nuclear RNA fractions from transformed cells were isolated and treated with a purified 3' exonuclease for various lengths of time before hybridisation to SV40 DNA. The relative level of hybridisation of exonuclease treated fractions should provide information on the approximate location of virus specific sequences. In addition, degraded pre-mRNA fractions were chromatographed on HAP in order to isolate fractions containing terminal 5' triphosphates. [35, 36] These may be considered to be true 5' termini of pre-mRNA molecules. Hence the level of hybridisation of these sequences compared to control fractions should demonstrate the presence or absence of virus-specific sequences at the 5' end of pre-mRNA molecules.

## Materials and Methods

### a) Preparation of SV40 DNA

DNA extracted from AGMK cells lytically infected with SV40 was provided by Dr. P. Chumakov. The DNA was then centrifuged isopycnicly in 5 M CsCl, 300g/ml ethidium bromide for seventy hours at 40,000 rpm and 20°C in a Beckman SW40 rotor, using not more than 5 mg of DNA per tube. Three visible bands of DNA were apparent after centrifugation of which the upper two were discarded. Fractions were removed from the top of the gradient using a Pasteur pipette. The upper two bands are cellular DNA and SV40 DNA form 11, and the lower SV40 form 1 DNA. Ethidium bromide was removed from the SV40 form 1 fraction by repeated extraction with isoamyl alcohol, followed by extraction once with di-ethyl ether to remove traces of isoamyl alcohol. Two volumes of ethanol were then added to precipitate DNA, which was left overnight at -20°C. Precipitated CsCl was removed by repeated washings with 70% ethanol. The SV40 DNA was further purified by sucrose density gradient centrifugation. 5 to 20% sucrose gradients were prepared by the method of Stone,<sup>[37]</sup> and spun at 25,000 rpm in an SW40 rotor for 3.5 hrs at 20°C. Fractions were collected by suction from the bottom of the tubes, and their OD<sub>260</sub> measured. Those fractions containing the main peak corresponding to 21S SV40 DNA form 1 were pooled and stored under two volumes of ethanol at -20°C.

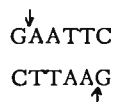
In order to assess the purity of the final fraction, electrophoresis on agarose gels was carried out. 1.4% agarose gels were prepared in buffer comprising 40 mM Tris acetate pH7.5, 1 mM EDTA, 5 mM sodium acetate. The gels were pre-electrophoresed, using the same buffer for half an hour, and samples of about 1 μg of DNA in 5 μl of

buffer containing 5% sucrose and 0.1% bromophenol blue as a tracking dye were applied to them. The samples were electrophoresed at 5 ma per gel for about two hours. Gels were then stained in 0.5% ethidium bromide for an hour and visualised under ultraviolet light. A single broad band of SV40 DNA form 1 was apparent in samples from the peak fractions of the sucrose gradient, with a relative mobility of about 0.6 compared to bromophenol blue.

The broadness of the peak is due to the variable amounts of super-coiling in the closed circular DNA.

b) DNA filter binding

Purified SV40 DNA form 1 must be converted to a linear molecule before denaturation to give single stranded DNA. The restriction endonuclease EcoR 1 cleaves SV40 at one site, [38] corresponding to the sequence



EcoR 1 was kindly provided by Dr. P. Chumakov. 50 µg of SV40 DNA form 1 were dissolved in 150 µl of water and 15 µl of buffer solution. The buffer comprised 400 mM Tris-HCl pH 7.4, 500 mM NaCl, 100 mM MgCl<sub>2</sub>, 100 mM mercaptoethanol. To the DNA solution was added 5 µl of EcoR 1 solution, and the solution incubated at 37°C for two hours. The reaction was stopped by the adjustment of the solution to 50 mM in EDTA. Agarose gel electrophoresis as described in the previous section was carried out to detect the progress of the reaction. Under the conditions described, all the SV40 form 1 band was converted to a sharper band corresponding to form 11 DNA with an Rf of about 0.4. Samples for electrophoresis

were boiled for a few minutes before application to the gels.

SV40 DNA form 11 was precipitated in two volumes of ethanol at  $-20^{\circ}\text{C}$  overnight, and resuspended at a concentration of less than  $1\mu\text{g}$  per ml in 0.01 SSC. The solution was boiled in a stoppered vessel for fifteen minutes, chilled in ice until cold, and boiled once again for five minutes. The solution was finally chilled in ice and immediately bound to millipore filters.

$0.45\mu$  - Millipore filters were soaked in a solution of 6 x SSC for fifteen minutes. They were then placed in teflon funnels, and each side of the filters washed with 20 ml of 6 x SSC. Ice-cold denatured DNA was then allowed to pass through the filters at  $4^{\circ}\text{C}$  without suction. In control experiments using tritiated DNA, after three passages of the DNA, 90% of the counts were absorbed. After several passages of the DNA, the filters were washed with 20 ml of 6 x SSC and dried overnight in petri dishes, taking care that only the edges of the filters were in contact with glass. Filters were then dried in a vacuum oven for three hours at  $80^{\circ}\text{C}$ . From control experiments using tritiated DNA, each filter bound about  $10\mu\text{g}$  of DNA. Approximate measurements of unlabelled DNA binding were made spectrophotometrically, assuming  $10D_{260}$  is equivalent to  $47\mu\text{g}$  of DNA.

c) Preparation of tritiated pre-mRNA

A transformed mouse kidney line was maintained in a medium composed of 50% Eagles medium, 50% 0.5% hydrolysed  $\alpha$ -lactalbumin. The medium was removed from cells and to each 250 ml roller bottle was added 2.5 ml of solution containing 0.5 mCi each of tritiated uridine, cytidine, adenine and guanine, 50% MEM with kanomycin, pH adjusted to 7.4 with HEPES buffer. Roller bottles were then rotated by hand in a warm room at  $37^{\circ}\text{C}$  for twenty minutes. Medium was decanted, and 20 ml of a buffer comprising 10 mM sodium acetate

pH 5.4, 0.15 M NaCl was added to each roller bottle. Cells were suspended by means of a rubber policeman and vigorous shaking. The buffer was then made 1% in SDS and immediately transferred to a flask containing one volume of buffer-saturated redistilled phenol. The flask was then heated at 65°C with vigorous shaking for three minutes, then chilled in ice. The contents were centrifuged at 5,000 rpm for fifteen minutes at 4°C. The aqueous layer was removed and two volumes of phenol added to it. The mixture was then shaken at 4°C. The phenolic layer was discarded and the interface further extracted with 20 ml sodium acetate buffer pH 5.4 and 20 ml of phenol as described above. The aqueous layer was combined with that previously extracted. Once again, the phenolic layer was discarded and the interface extracted with 5% redistilled m-cresol at 85°C as described for the phenolic extractions. All the combined aqueous layers were then shaken at 4°C for one hour with an equal volume of phenol. The suspension was then centrifuged at 5,000 rpm for fifteen minutes at 4°C, and the aqueous layer retained. Further extractions using a mixture of phenol and chloroform (1:1), and then chloroform alone, were carried out on the aqueous layer, which was finally treated with 2.5 volumes of redistilled ethanol and stored overnight at -20°C. The rationale behind this extraction procedure has been described by Scherrer. [34]

The precipitated tritiated RNA was then isolated by centrifugation for one hour at 5,000 rpm at 4°C, and further precipitated with ethanol twice more, in order to remove contaminating SDS. The precipitate was then resuspended in 2.5 ml of buffer comprising 100 mM Tris-HCl pH 7.4, 10 mM MgCl<sub>2</sub> and incubated at 25°C for twenty minutes in the presence of five units of Worthington DNase.

The material was then fractionated on sucrose density gradients



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The material was then fractionated on sucrose density gradients

in the presence of SDS. Aggregation was apparent when identical samples of RNA treated or untreated with DMSO were compared. Hence DNase treated samples were made 90% in DMSO, and heated to 35°C for ten minutes. The samples were then diluted three times with 0.01 M HEPES pH 7.4, 0.1% SDS .01 M EDTA buffer, and centrifuged in 15 to 30% sucrose gradients in 0.2% SDS, 10 mM EDTA in an SW27 rotor at 27,000 rpm for five hours at 20°C. Ribosomal RNAs were used as markers. Gradients were fractionated dropwise by suction from the bottom of each tube into about thirty fractions. 5 $\mu$ l aliquots from each fraction were applied to glass filter discs, which were dried, washed with 5% TCA, and then counted after further drying in a toluene based scintillant. Gradients containing marker RNAs were scanned in a recording spectrophotometer at 260 nm, material being sucked from the bottom of the tube through a flow cell at a constant velocity. Fractions of various molecular weight ranges were pooled, precipitated with 2.5 volumes of ethanol, and stored at -20°C.

d) Exonuclease treatment

Ehrlich ascites 3' exonuclease (E.C.3.1.4. ) was kindly provided by Dr. P. Chumakov, prepared by the method of Lazarus and Sporn.<sup>[40]</sup> The final preparation had about 0.02% endonuclease activity as judged by its behaviour with rRNA.

The enzyme was assayed in a solution containing 40 mM Tris-HCl pH 7.4 4 mM MgCl<sub>2</sub>, 0.3 mM DTT, 40 mM KH<sub>2</sub>PO<sub>4</sub>, 20 - 100 $\mu$ g/ml of diethyl pyrocarbonate treated BSA, 1 mg/ml [<sup>3</sup>H] poly A, and 0.1 to 0.75 units of enzyme per ml at 37°C. The reaction was stopped by the adjustment of the solution to 5% TCA, samples were chilled, spun on a bench centrifuge, and aliquots from the supernatants of each sample dried on glass fibre discs and counted in a toluene-based scintillation fluid. One unit of activity is defined as that which

hydrolyses  $1 \mu\text{M}$  of poly A per hour at  $37^\circ\text{C}$ .

Exonuclease treatment of pre-mRNA was carried out on various fractions isolated by density gradient centrifugation as previously described. Pre-mRNA fractions were first resuspended in 0.5 ml of 50 mM NaCl, 1 mM EDTA pH 7.0, and chromatographed on a Sephadex G25 column (8 x 0.5 cm) collecting fractions of about 0.7 ml, in order to remove SDS. A number of control experiments were carried out to optimise conditions for exonucleolytic degradation. Initial rates of breakdown were assessed with varying concentrations of enzyme to determine the level of enzyme which saturated all free 3' OH groups.

The time course of the reaction at saturating levels of enzyme was followed by determining the level of radioactivity solubilised in 5% TCA. In addition, aliquots at various stages of the reaction were removed, made 0.2% in SDS, and analysed by agarose gel electrophoresis in order to detect possible endonucleolytic cleavage. About 5 to  $10 \times 10^4$  cpm of [ $^3\text{H}$ ] RNA were applied to a 1% agarose gel with bromophenol blue as tracking dye. Electrophoresis buffer comprised Tris-acetate pH 7.4, 1 mM EDTA, 5 mM sodium acetate. Ribosomal RNAs were used as markers. After electrophoresis, the marker gels were stained in 0.5% ethidium bromide for thirty minutes, and visualised under UV light at 254 nm. About  $2 \mu\text{g}$  of rRNA was used per gel, and Rfs for 18 s and 28 s rRNA were 0.68 - 0.84 with respect to bromophenol blue. Gels containing tritiated pre-mRNA were sliced into thirty pieces, each slice being placed on a 2 cm square of filter paper, to which was added 0.2 ml of water. The gel slices were then frozen at  $-70^\circ\text{C}$  and thawed, then dried and counted in a toluene based scintillant.

The possible inhibitory effect of trace contamination with DNA was assessed by following the reaction in the presence of  $500 \mu\text{g/ml}$

of mouse nuclear DNA. No inhibition was observed. Exonuclease treatment was carried out on pre-mRNA samples prior to hybridisation in the following manner. Pre-mRNA was again treated with Worthington DNase in exonuclease assay buffer for thirty minutes at 25°C. The solution was then divided into equal fractions, each containing at least 10<sup>7</sup> cpm of tritiated RNA which were treated with a saturating amount of exonuclease at 37°C for different times. At selected time intervals, fractions were made 0.2% in SDS, and aliquots taken for the determination of TCA soluble radioactive material, and for electrophoretic analysis on agarose gels as described above. The rest of the material was then precipitated with 2.5 volumes of ethanol at -20°C overnight, and isolated by centrifugation at 5,000 rpm for one hour at 4°C.

e) Hybridisation procedure

Hybridisation was carried out at 65°C in 6 x SSC 0.2% SDS for eighteen hours or at 41°C in 7 M urea, 2 x SSC 0.2% SDS for forty-eight hours. Each stoppered vial contained one eighth of a blank filter, and one eighth of a filter containing about 1 µg of SV40 DNA. Tritiated RNA samples were dissolved in 0.15 ml of the appropriate medium, and incubated with the filters in a small stoppered vial. Each sample contained at least 5 x 10<sup>6</sup> cpm of [<sup>3</sup>H] RNA, of specific activity in the region of 4 x 10<sup>6</sup> cpm/µg. After incubation, filters were thoroughly washed with 50 ml of 2 x SSC at 20°C. The filters were then suspended in 7 M urea, 2 x SSC 0.2% SDS at 41°C for two hours, then washed again with 2 x SSC. This washing procedure was repeated three times. Filters were then suspended in 2 x SSC and incubated at 65°C for thirty minutes. Filters were then treated with 0.5 µg/ml of pancreatic ribonuclease in 2 x SSC at 37°C for forty-five minutes. The pancreatic ribonuclease was previously

incubated at 100°C for two minutes to remove possible contaminating DNases. Filters were then washed with 50 ml 2 x SSC, 50 ml 5% TCA and 50 ml 16.5% TCA. After this washing, the filters were dried, and counted in a toluene based scintillation fluid.

f) Purification of true 5' pre-mRNA fragments

Bajszar<sup>[35]</sup> has fractionated oligonucleotide fragments of from 50 to 100 nucleotides in length on the basis of their 5' termini by HAP chromatography. Those fractions with 5' terminal triphosphates are eluted from HAP columns at higher salt concentrations than oligonucleotides terminating in monophosphates, and are considered to be true pre-mRNA 5' fragments.

Tritiated pre-mRNA, extracted as described previously, was sheared to approximately 4 s fragments by alkaline hydrolysis. To pre-mRNA dissolved in 1 mM EDTA, 0.1% SDS was added 0.1 volume of 1N NaOH. The solution was left at room temperature for twenty minutes, and at 4°C for ten minutes. The solution was then neutralised with 5% acetic acid and fractionated on a 5 to 20% sucrose gradient containing 1 mM EDTA 0.1% SDS for eighteen hours at 40,000 rpm and 4°C in an SW40 rotor, using tRNA as a marker. Gradients were fractionated and counted as described in section C. Material was then precipitated at -20°C overnight with 2.5 volumes of ethanol, resuspended in 0.02 M phosphate buffer pH 6.8 and applied to a 10 x 1.5 cm column, containing 50% Biorad HAP, 50% cellulose, which had previously been washed with 200 ml of 0.04 M phosphate buffer pH 6.8. After sample application, the column was washed for one hour with more buffer at a flow rate of about 10 ml per hour. A linear gradient of 0.04 to 0.32 M phosphate buffer, pH 6.8, 0.1% SDS was then applied to the column. Fractions of about 1 ml were collected and phosphate concentration calculated using a refractometer.

5  $\mu$ l aliquots from each fraction were dried on glass fibre discs and counted in a toluene based scintillant. Material eluting at a phosphate concentration of about 0.12 M was pooled, concentrated, and adjusted to 0.04 M phosphate, using an ultrafiltration device with an amicon PM10 membrane. This material was rechromatographed in an identical manner, a discrete peak being observed at a concentration of about 0.1 M phosphate. This material was once again chromatographed to give a further purification of the 5' terminal pre-mRNA fractions, which were pooled and stored at  $-20^{\circ}\text{C}$  under 2.5 volumes of ethanol. Material eluting earlier from the HAP column was also stored for control purposes.

### Results and Discussion

The distribution of radioactivity during the phenolic extraction of rapidly labelled RNA is shown in Table 1. A high proportion of TCA-insoluble counts are retained in the aqueous layer during the extraction procedure.

TABLE 1

Extraction number	Total counts per minute per 5 $\mu$ ls	Counts per minute per 5 $\mu$ ls in aqueous extract
1	89,677	42,218
2	24,430	13,330
3	3,248	1,529

Data on the size distribution of rapidly labelled RNA are presented in diagrams 4 and 5. Material from regions A and B was used for hybridisation studies, as the molecular size of these fractions was considerably higher than that of the whole SV40 genome. Preparations of labelled pre-mRNA more than one week old, and low molecular weight fractions isolated by sucrose gradient centrifugation after DMSO treatment were degraded to approximately 4S size and used for HAP chromatographic isolation of 5' pre-mRNA termini, as previously described. Figure 6 shows the size distribution of alkaline treated RNA, and the elution profiles of successive HAP chromatography steps are shown in Figure 7. Unfortunately, too little material was obtained for hybridisation studies by this technique, so that radioiodination of purified 5' fragments had to be carried out. This work was carried out by Chumakov and Mantieva on my purified material,

FIGURE 4

Typical size-distribution of rapidly labeled nuclear RNA. Preparative sucrose density gradients were run according to the text. Material in regions A and B was used for hybridisation studies.



FIGURE 4

Typical size-distribution of rapidly labelled nuclear RNA. Preparative sucrose density gradients were run according to the text. Material in regions A and B was used for hybridisation studies.

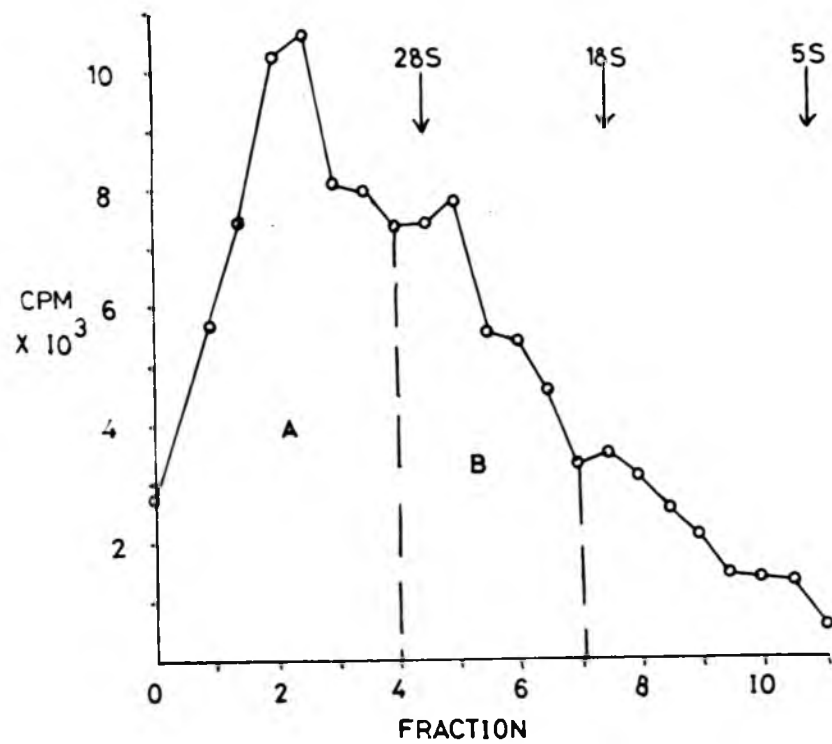


FIGURE 5

Size-distribution of rapidly labelled nuclear RNA,  
as determined by analytical gel electrophoresis  
according to the text.

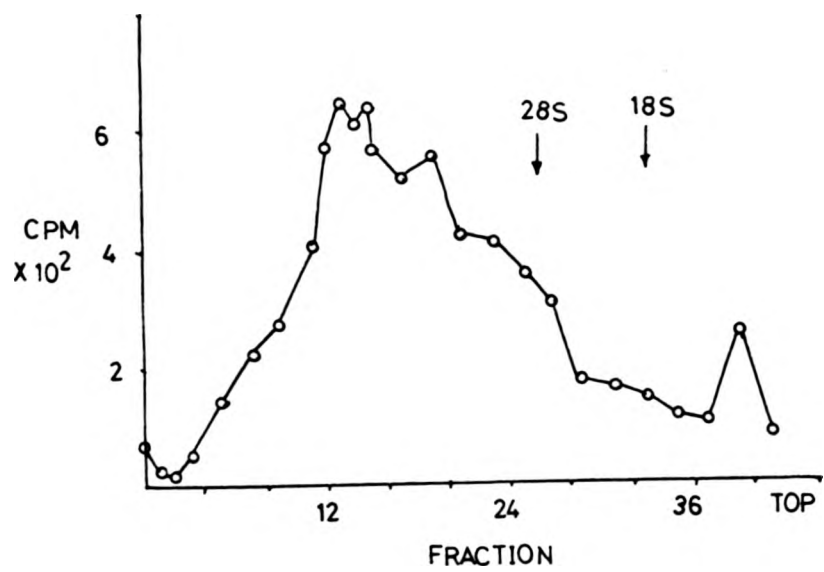


FIGURE 6

Alkaline treated pre mRNA, fractionated by sucrose density centrifugation according to the text, prior to HAP chromatography.

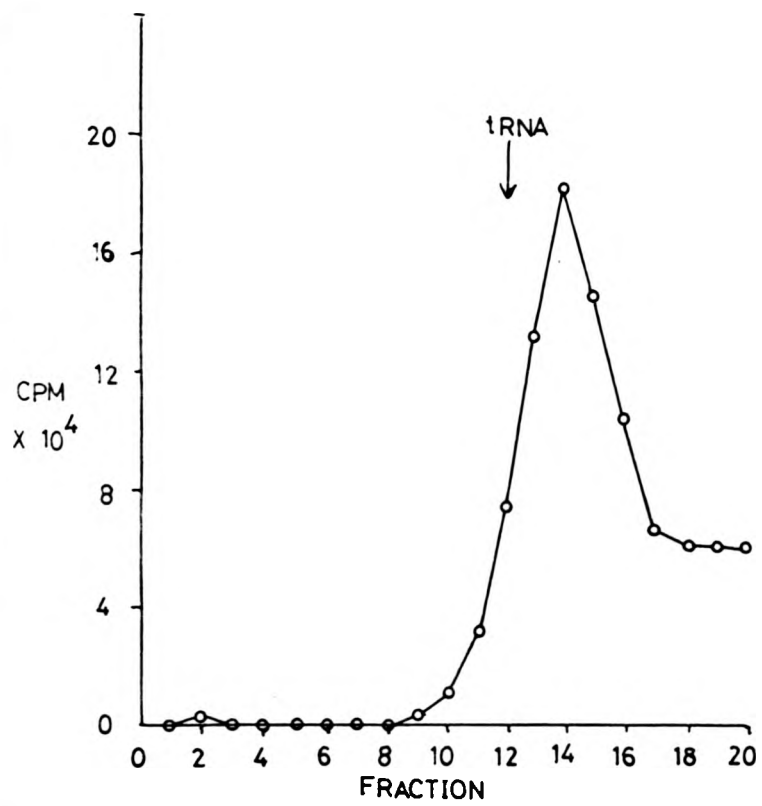
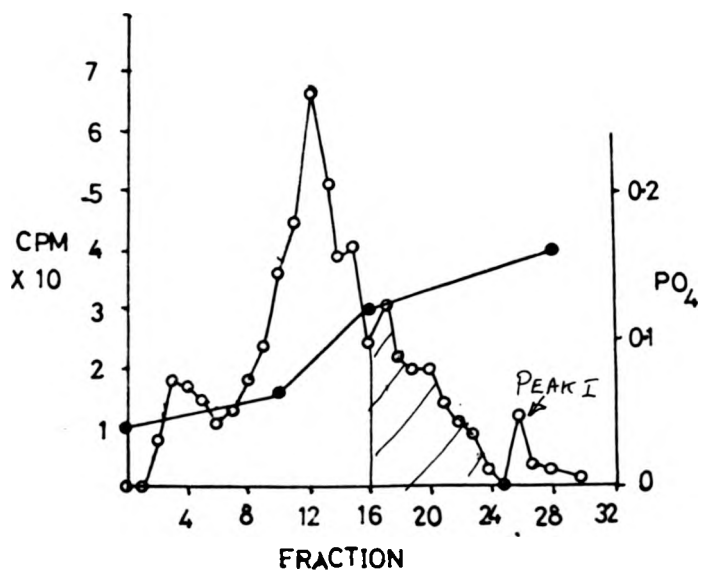
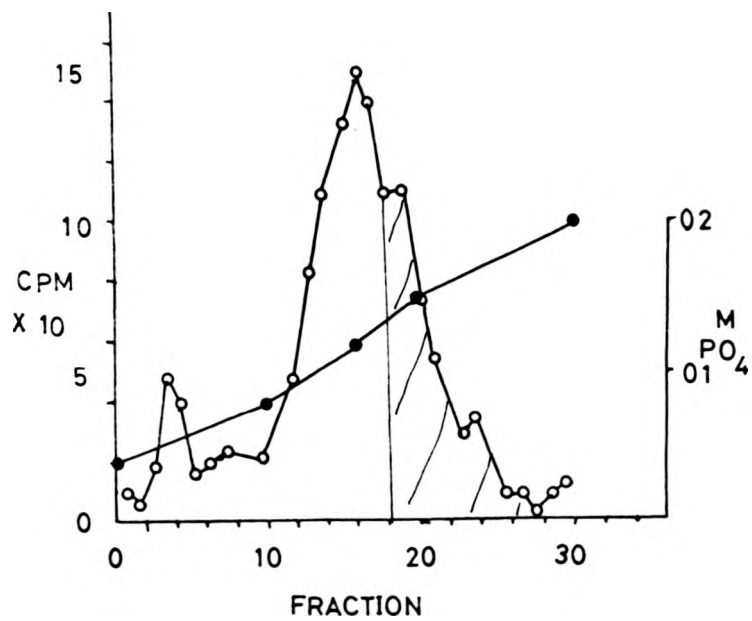
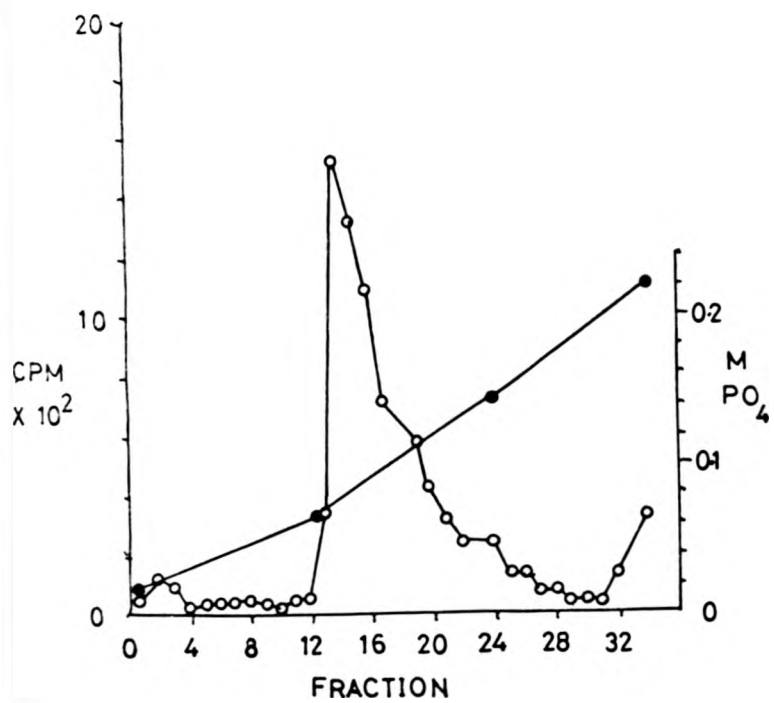


FIGURE 7

Successive HAP fractionations of 4 s pre-mRNA.  
Shaded regions were rechromatographed.  
Peak 1 was used for hybridisation studies, after  
radio-iodination. [46]







and preliminary hybridisation data carried out by them suggests that purified 5' pre-mRNA fragments are enriched for SV40 specific sequences. Two to three times as much hybridisation occurred with these fractions, as with control fractions of a similar specific activity.

Control experiments using exonuclease 111 kindly provided by Dr. P. Chumakov are presented in Figures 8 and 9. Data on the effect of contaminating DNA on the activity of the exonuclease are presented in Table 3. Gel electrophoretic profiles of exonuclease treated pre-mRNA are presented in Figure 10. The effect of contaminating endonuclease activity can be clearly seen in Figure 11 where an impure preparation of exonuclease 111 was inadvertently used.

The results of hybridisation of fractions treated with exonuclease are presented in Figure 12 and Table 2. The level of hybridisation shown by untreated pre-mRNA is in agreement with the figure found by Mantieva and Georgiev during their UV-irradiation experiments. The results of a single experiment (Figure 12) show a correlation between the processive degradation of the 3' end of pre-mRNA and an enrichment of SV40 sequences in the remaining RNA. Another experiment confirmed this, and recently Chumakov, using pre-mRNA fractions sheared to equal sizes, has obtained an identical result.<sup>[41]</sup> The evidence thus suggests that SV40 specific sequences are distributed towards the 5' end of pre-mRNA. However, each of the three approaches to the problem of the location of viral sequences in pre-mRNA suffers from a number of faults. In particular, the hybridisation data are suspect, as specific activity may vary down the pre-mRNA molecule, and the low input of RNA may mean that all sequences may not be fully bound under the conditions described here. Work to eliminate these problems is

TABLE 2

Time of incubation with exonuclease	% TCA soluble material	% hybridisation
0	7.9	0.0142
30	8.9	0.0133
60	18	0.0219
90	30	0.0254

TABLE 3

Effect of DNA and Protein on exonuclease degradation of RNA

Reaction mixture	Arbitrary Units
- Enzyme	0
+ Enzyme	350
+ Enzyme + 100 $\mu$ g/ml BSA	355
+ Enzyme + 100 $\mu$ g/ml DNA	358
+ Enzyme + 100 $\mu$ g/ml BSA + 100 $\mu$ g/ml DNA	350

FIGURE 8

Exonuclease produced TCA soluble counts from  
rapidly labelled nuclear RNA.

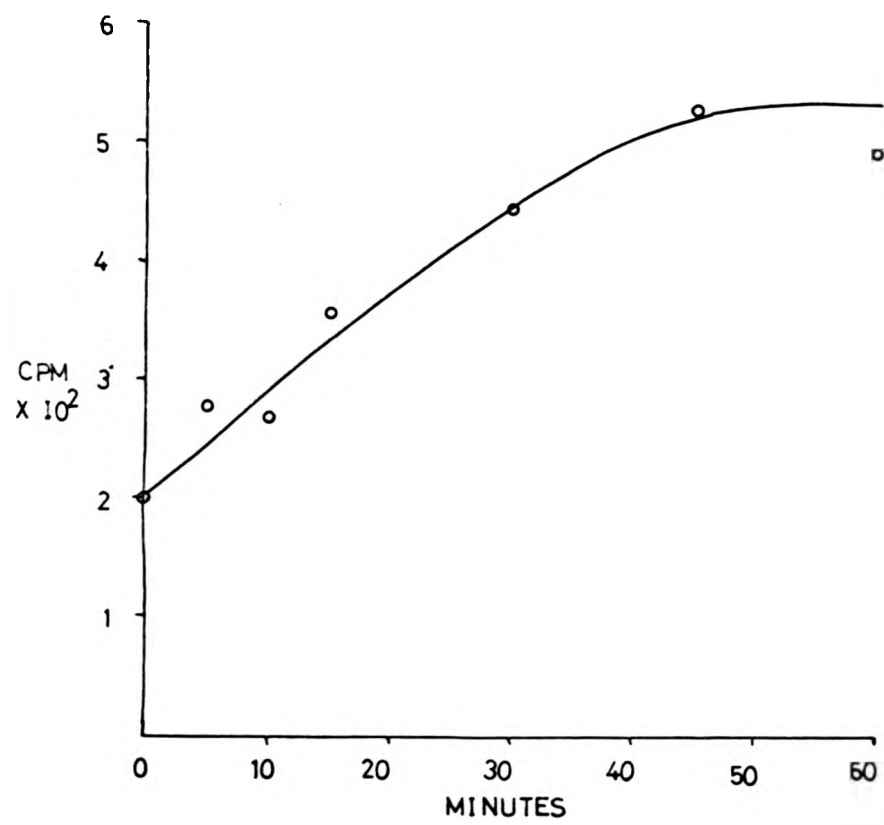


FIGURE 9

Determination of saturating level of exonuclease:  
initial velocity with variable amounts of enzyme.

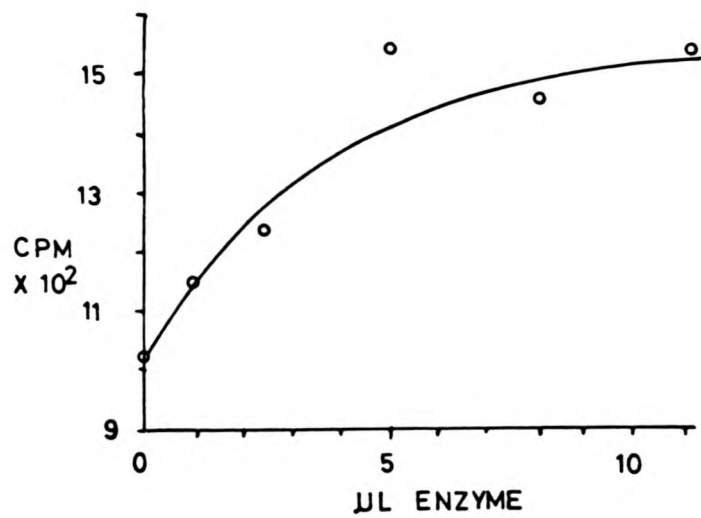


FIGURE 10

Gel electrophoresis, according to the text, of exonuclease •  
treated pre-mRNA and a control sample. ○



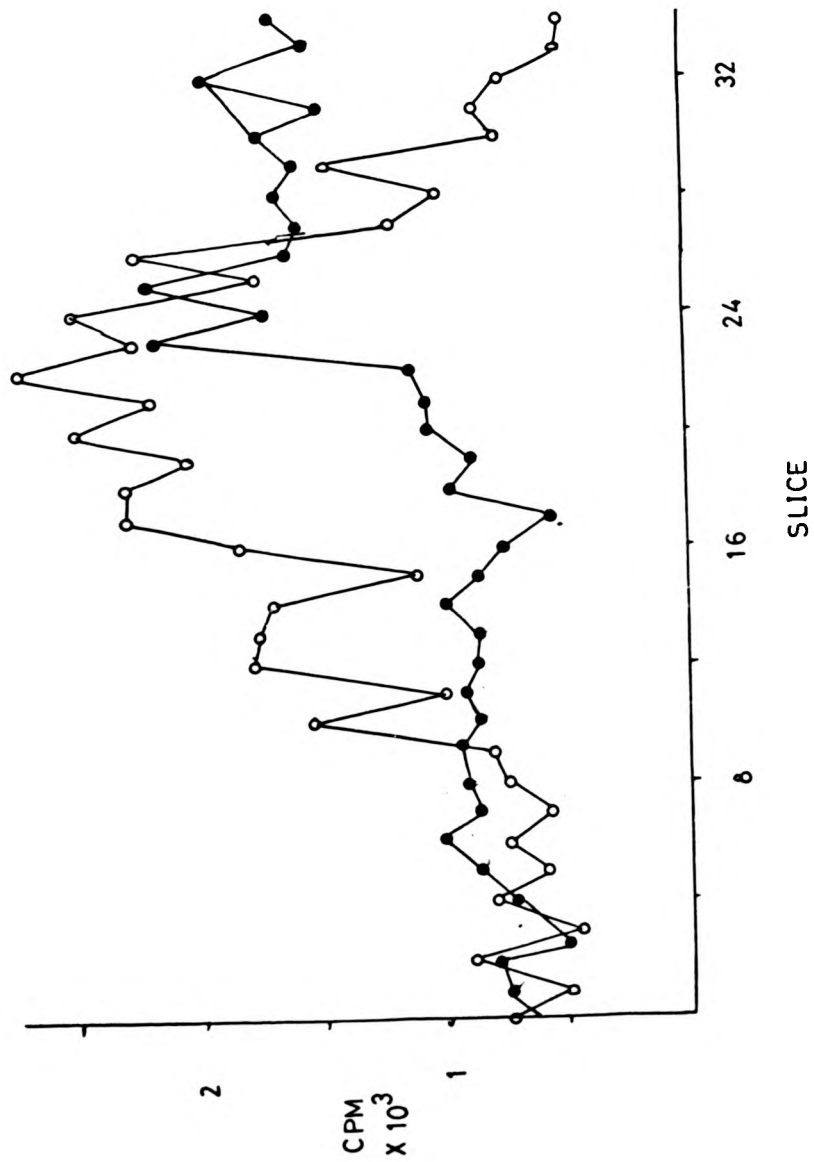


FIGURE 11

Effect of endonuclease contamination on pre-mRNA  
degradation by exonuclease.

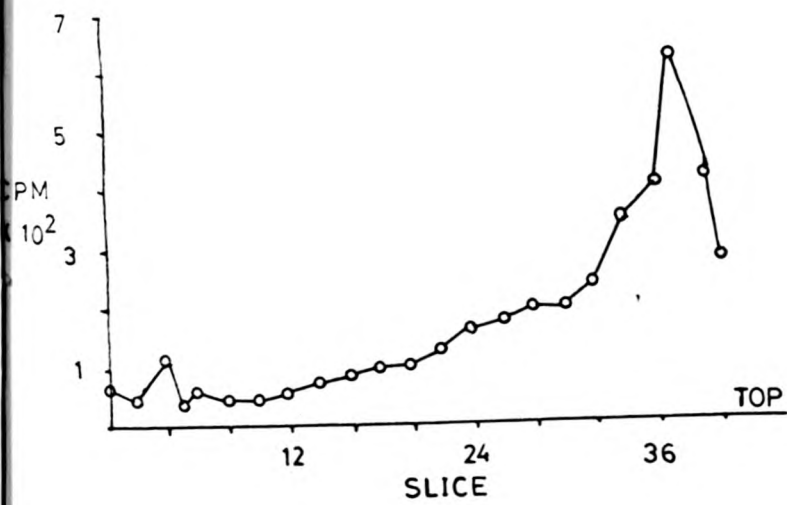
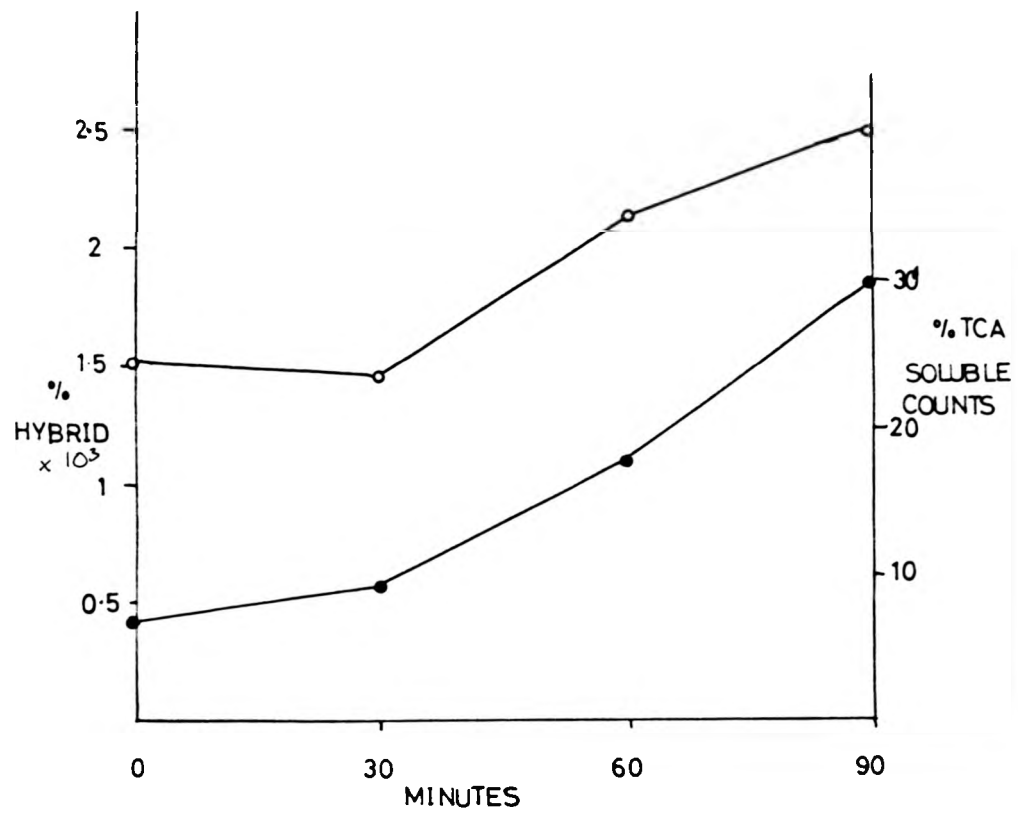


FIGURE 12

Percentage hybridisation of pre-mRNA to SV40  
DNA, after exonuclease treatment, and  
percentage TCA soluble counts produced by  
exonuclease with time.



now being carried out in Georgiev's laboratory. Nevertheless, in qualitative terms, it is difficult to dismiss the hybridisation data from all three experiments, each of which depended on comparative levels of hybrid formation between treated material and untreated controls.

Other objections may be made to each experiment. UV irradiation may lead to the activation of a viral promoter, for instance, so that larger amounts of viral RNA are synthesised after irradiation. The premature termination of transcription may be more likely at certain sites, so that what appears to be an enrichment of viral sequences may be caused by a relatively lower level of expression of other RNA sequences. The exonuclease treatment experiment also suffers from the fact that the enzyme is sensitive to the secondary structure of its substrate,<sup>[42]</sup> and so figures for the percentage degradation of pre-mRNA derived from the appearance of TCA soluble radioactivity, may not present a true picture for an individual pre-mRNA species.

The isolation of supposed 5' termini of pre-mRNA by HAP chromatography may also be questioned. Assuming such a fraction does contain true nascent pre-mRNA fragments, hydrolysis of di- or triphosphate termini during the isolation procedure may lead to considerable contamination of control fractions with 5' di- or triphosphate terminating oligonucleotides. The recently discovered phenomenon of capping<sup>[43]</sup> also complicates the purification procedure, although Bajszar has demonstrated that capped 5' ends do not co-elute with or effect the purification of 5' triphosphate ends.<sup>[44]</sup> The coincidence of the results is nevertheless striking, and the fact that the levels of hybridisation are of a plausible and reproducible size is encouraging. A major cause for concern, however,

is that a fragment of repeated sequence host DNA may be present in the SV40 DNA used for hybridisation. As repeated sequences are found at the 5' end of pre-mRNA, apparent enrichment of virus specific sequences at the 5' end of pre-mRNA would be observed. The possibility that host DNA sequences are present in the genome of SV40 is suggested by the observation of positive levels of hybridisation between SV40 and uninfected cells.<sup>[45]</sup> The use of low-titre cloned stock virus in these experiments, together with extremely low levels of hybridisation in control experiments with 3T3 cells<sup>[41]</sup> obviates these objections to a considerable extent.

With the above provisos, the apparent absence of viral sequences at the 3' end of pre-mRNA molecules has a number of implications. Either SV40 RNA processing differs significantly from that of endogenous RNA species, or current theories of eukaryotic mRNA processing are incorrect. The expression of DNA sequences which are interposed between repetitive and supposedly regulatory host sequences may have significance for the mechanism of viral transformation, perhaps disturbing the level of transcription and the stability of the RNA encoded for by regulatory DNA. Much work obviously remains to be done, the difficulties of which are considerable.

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Thermostable polynucleotide phosphorylases from *Bacillus stearothermophilus*  
and *Thermus aquaticus*

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Received 21 November 1975

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ABSTRACT

Polynucleotide phosphorylase from *Bacillus stearothermophilus* has been purified to homogeneity. Polyacrylamide gel electrophoresis run under denaturing conditions indicates that the enzyme is a tetramer with subunits of apparent molecular weight 51,000 daltons. A partial purification of polynucleotide phosphorylase from *Thermus aquaticus* has also been effected. The two enzymes show similar catalytic properties, which differ little from those of mesophilic polynucleotide phosphorylases. The use of thermostable polynucleotide phosphorylases for in vitro nucleic acid synthesis is discussed.

INTRODUCTION

Polynucleotide phosphorylases (E.C.2.7.7.8.) of the thermophilic organisms *Bacillus stearothermophilus* and *Thermus aquaticus* have been studied in the hope of easily polymerising modified nucleotide diphosphates. Although the substrate specificities of the two enzymes are similar to those reported for mesophilic enzymes, their thermal stability does present advantages, for instance in the synthesis of polyriboguanlyic acid from GDP or in the copolymerisation of GDP with other nucleotides to give polymers of base composition similar to the input ratio of nucleotides<sup>1</sup>. Preliminary structural studies have been carried out on the homogenous *B. stearothermophilus* enzyme, which can be readily obtained as a single active protein species. In contrast, purification of the enzyme from other sources e.g. *Micrococcus luteus* usually leads to the isolation of a number of different active species, presumably as a result of proteolysis of the original enzyme.<sup>2</sup>

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### MATERIALS

B.stearothermophilus (NCIB 8924) and T.aquaticus were supplied as freeze dried pastes by MRE Porton, and stored at  $-20^{\circ}\text{C}$  until used. Polynucleotides were supplied by P.L. Biochemicals Inc. Nucleotide diphosphates and alkaline phosphatase (E.C.3.1.3.1.) were supplied by Boehringer Corporation. Other enzymes were supplied by BDH, radioactive materials were obtained from the Radiochemical Centre, Amersham. Dimethyl suberimidate was synthesised as described by Davies and Stark<sup>3</sup>.

### METHODS

Protein was assayed by the Biuret or Lowry methods.<sup>4</sup> Purified samples of proteins were assayed spectrophotometrically<sup>5</sup>. Polymerisation assays utilised the colorimetric determination of phosphate released with time<sup>6</sup>, and phosphorolysis was assayed by the method of Klee.<sup>7</sup> One unit of enzyme activity is defined as that which will polymerise one  $\mu\text{mole}$  of ADP per hour. Polymerisation assays were carried out at  $60^{\circ}\text{C}$  in a mixture containing 50mM Tris-HCl pH 9.2, 20mM ADP, 1mM DTT, 1mM EDTA, 0.6M KCl, 7.5mM  $\text{MgCl}_2$ , and 100 $\mu\text{M}$  oligo (A) primer. Phosphorolysis assays were carried out at  $60^{\circ}\text{C}$  in a mixture containing 50mM Tris-HCl pH 8.2, 1mM DTT, 1mM EDTA, 0.6M KCl, 100 $\mu\text{M}$  poly A and 10mM [ $^{32}\text{P}$ ]-labelled  $\text{KH}_2\text{PO}_4$  with a specific activity of about  $10^5$  cpm per  $\mu\text{mole}$ .

Oligo (A) primer was prepared by treating poly (A) with 0.1 M NaOH at  $25^{\circ}\text{C}$  for 3 hr., bringing the solution to pH 8.0 with acetic acid and treating with alkaline phosphatase at  $37^{\circ}\text{C}$  for one hr. (50 units of enzyme per mg. of poly (A)). The solution was then deproteinised with isoamyl alcohol:chloroform (5:2 v/v), and fractionated on a Sephadex G10 column equilibrated with 50mM Tris-HCl pH 8.2, 0.2M KCl. Samples were stored at  $-20^{\circ}\text{C}$ .

### ENZYME PURIFICATION

B.stearothermophilus was grown under the growth conditions described by Sargent.<sup>8</sup> T.aquaticus growth conditions were as described by Brock and Freeze<sup>9</sup> with the exception of the substitution of tris(hydroxymethyl)aminomethane for nitrilotriacetic acid, resulting in a considerable slowing of the growth rate. Freshly harvested or freeze dried B.stearothermophilus (25g) was suspended in Buffer A (Tris-HCl 50mM pH 8.2, 1mM EDTA, 1mM DTT (100 ml) and broken by treatment with lysozyme and EDTA.<sup>10</sup> Cell debris was centrifuged off, and saturated ammonium sulphate solution

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added to precipitate those proteins insoluble between 35 and 55% ammonium sulphate. This fraction was isolated by centrifugation then resuspended in buffer A and applied to a TEAE-cellulose column (30 x 2.5 cms) which was eluted with a linear gradient of 0 to 0.5 M KCl in buffer A. The active fractions, eluting at a concentration of about 0.28M KCl were pooled, concentrated and desalted in a Bio-Rad hollow fibre device. The protein was then applied to a DEAE-Sephadex A50 column (40 x 1.5 cms), and eluted with a linear gradient of 0 to 0.5 M KCl in buffer A. Active fractions were pooled, precipitated with ammonium sulphate, and applied in a minimal volume of buffer A to an LKB AcA 34 Ultrogel column (60 x 2.5 cms). Active fractions were combined and stored at -20°C. The purification of the T.aquaticus enzyme was essentially the same, except that cell lysis was effected by ultrasonication at full power for thirty seconds in an MSE 150 watt ultrasonicator. Material purified to the stage after TEAE-cellulose chromatographs was used for preliminary characterisation. The presence of nucleases, phosphatases and adenyly kinase activity in purified preparations was assessed. Nuclease activity was detected by measuring the production of TCA-soluble material from [<sup>14</sup>C]-labelled poly(A) or poly(C) in a phosphorolysis assay mixture containing no inorganic phosphate. Phosphatase activity was measured under polymerisation assay conditions, with no metal co-factor present. Adenyly kinase activity was measured using a polymerisation assay mixture containing [<sup>14</sup>C]-ADP. Reaction products at various time intervals were chromatographed on Whatman 4 paper in an isobutyric acid/NH<sub>4</sub>OH/H<sub>2</sub>O (66/1/33) solvent system. The ratio of counts co-chromatographing with ADP to those co-chromatographing with AMP was determined.

Approximate molecular weights for native B.stearothermophilus and T.aquaticus enzymes were determined by gel exclusion chromatography on an LKB AcA 34 Ultrogel column (80 x 2.5 cms) equilibrated with buffer A, using lysozyme, haemoglobin and glutamate dehydrogenase as molecular weight markers. Comparison of molecular weights with E.coli polynucleotide phosphorylase was carried out by density gradient centrifugation in a 5 to 20% sucrose gradient at 30,000 rpm for 22 hr. in an MSE 3 x 25 ml rotor. Samples were run separately or as admixtures.

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Standard polyacrylamide gel electrophoresis was carried out in phosphate, Tris-glycine or borate-acetate buffer systems, using 7.5% gels. Denaturing gels were run in the presence of SDS and mercaptoethanol<sup>12</sup>, molecular weights being computed by comparison with bovine serum albumin, yeast mitochondrial F<sub>1</sub> ATPase and lysozyme as markers.

Isoelectric focussing was carried out by the method of O'Farrell<sup>13</sup> and N-terminal determinations were carried out by the method of Weber.<sup>14</sup>

### RESULTS

Enzyme preparations were carried out on bacteria harvested in the late logarithmic phase of growth. However, the specific activity of the enzyme was highest in the early logarithmic phase, and varied with growth in a manner analogous to that observed for E.coli.<sup>15</sup> The specific activity of the T.aquaticus enzyme was also twice as high in the early stages of growth.

Crude extracts of the B.stearothermophilus and T.aquaticus enzymes were primer independent. Early B.stearothermophilus enzyme preparations using A25 Sephadex produced primer independent enzyme, which could be converted to primer dependency by gel filtration on a Sephadex G200 column. However, Sephadex A50 chromatography produced primer dependent enzyme as a broad peak with the leading and trailing edges showing different requirements for primer. A four fold stimulation of CDP polymerisation was achieved when an oligo (A) primer was added to the trailing fractions of the peak whereas a fifty fold stimulation was achieved with the leading fractions. This suggests that an easily dissociable factor is responsible for conferring primer independency on the enzyme. As the factor is present in the same fractions as polynucleotide phosphorylase eluted from an A25 column, but is separated from the enzyme by A50 Sephadex chromatography, a molecular weight of between  $1$  and  $2 \times 10^5$  daltons for the factor is suggested. By recombining fractions treated with nucleases or proteases with primer dependent enzyme, we attempted to determine the nature of this factor. However, no reconstitution of primer independency could be observed using this approach. The T.aquaticus enzyme also showed primer dependency on purification past the stage of TEAE-cellulose chromatography. Purified polynucleotide phosphorylase from B.stearothermophilus ran as a single band on polyacrylamide gel electrophoresis. The protein had a specific activity of 600 units per mg, an extinction coefficient of  $E_{280}^{1\%} = 5.68$

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and a 280/260nm absorbance ratio of 1.68, indicating the absence of contaminating nucleic acids.<sup>4</sup> No phosphatase, nuclease or adenyl kinase activity could be detected in the final preparation. The T.aquaticus enzyme purified to the stage of TEAE-cellulose chromatography was contaminated with nucleic acid and showed low levels of adenyl kinase activity.

The molecular weight of the B.stearothermophilus enzyme was  $5.1 \times 10^5$  daltons as judged by behaviour on gel filtration and density gradient centrifugation, the behaviour of the enzyme being similar to that of the E.coli polynucleotide phosphorylase purified by a similar procedure. Impure T.aquaticus enzyme had a molecular weight of more than  $4 \times 10^5$  daltons as judged by gel filtration. Gel electrophoresis of B.stearothermophilus polynucleotide phosphorylase under denaturing conditions showed a single protein band of molecular weight  $5.1 \times 10^4$  daltons. Cross-linking of the protein with dimethyl suberimidate<sup>3</sup> prior to electrophoresis led to the appearance of three more bands of lower mobility in positions corresponding to a dimer, trimer, and tetramer of the monomeric subunit. We therefore consider the enzyme to be tetramer of subunits of molecular weight  $5.1 \times 10^4$  daltons. N-terminal analysis by the method of Weber<sup>12</sup> showed the presence of a single dansylated amino acid, identified as methionine by comparison with standards. This observation provides evidence for the identical nature of the subunits. Isoelectric focussing in polyacrylamide gels<sup>13</sup> showed the isoelectric point of the pure enzyme to be 4.1.

#### CATALYTIC STUDIES

Both enzymes showed pH optima for polymerisation of 9.2 and for phosphorolysis of 8.2. The optimal nucleotide to magnesium ratio is about 3:1 for all nucleotides with the exception of GDP which is polymerised optimally at a ratio of 1:1. In these aspects both enzymes resemble polynucleotide phosphorylases previously characterised from other sources<sup>16</sup>. However, the optimal temperature for crude or pure preparations of the B.stearothermophilus enzyme is 69°C, whilst crude T.aquaticus enzyme functions optimally at about 80°C, although the partially purified material has an optimum of 73°C. In common with other thermostable enzymes, B.stearothermophilus polynucleotide phosphorylase was activated by salt although

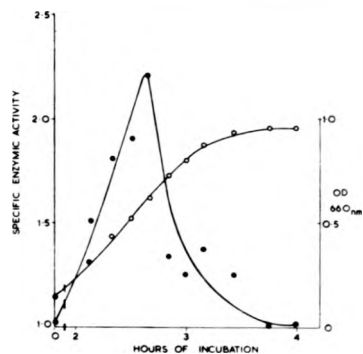


FIGURE 1  
VARIATION OF SPECIFIC ACTIVITY OF B. STEAROTHERMOPHILUS  
POLYNUCLEOTIDE PHOSPHOROLYSIS WITH GROWTH

Cell numbers were estimated from the turbidity at 660 nm. Aliquots of cell suspension were lysed ultrasonically and assayed for both phosphorolysis activity and protein concentration as described in the text.

●—●—●—●—● phosphorolysis activity per mg protein  
 ○—○—○—○—○ turbidity

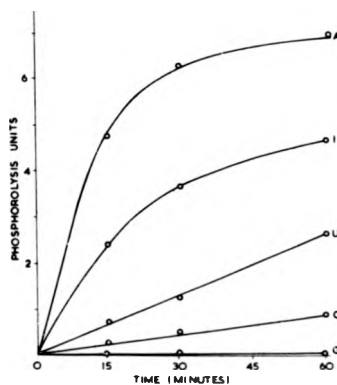


FIGURE 2  
PHOSPHOROLYSIS OF POLYNUCLEOTIDES UNDER STANDARD CONDITIONS  
BY B. STEAROTHERMOPHILUS POLYNUCLEOTIDE PHOSPHORYLASE

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FIGURE 3  
PHOSPHOROL  
BY T. AQUATI



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the crude T.aquaticus enzyme was not. We observed an interesting effect of  $\text{Ca}^{++}$  ions on the polymerisation activity of the B.stearothermophilus enzyme, which may be of some physiological significance. Although at millimolar concentrations  $\text{Ca}^{++}$  ions inhibit both phosphorolysis and polymerisation assays, at concentrations of about  $5\mu\text{M}$ , there is a selective activation of polymerisation by about three-fold. By analogy with the levels found in E.coli<sup>17</sup>, intracellular concentrations of  $\text{Ca}^{++}$  ions might be of this order.

The substrate specificities of the two enzymes varied through the purification procedure, becoming more selective with purification. Phosphorolysis data are presented in Figures 2 and 3. Neither enzyme showed polymerisation activity with the modified nucleotides  $\text{br}^9\text{ADP}$ <sup>18</sup>,  $\text{br}^8\text{GDP}$ <sup>18</sup> or 8-azido ADP<sup>19</sup> even in the presence of  $\text{Mn}^{++}$  ions, and all were found to be strong competitive inhibitors. Attempts to degrade processively sheared calf thymus or T4 DNA which had been previously treated with alkaline phosphatase were also unsuccessful. Hence the enzymes from thermophilic bacteria show a rather narrower range of substrate specificities compared with polynucleotide phosphorylases from mesophilic organisms.<sup>16</sup> Poly (A) is synthesised and degraded rapidly by the enzyme from B.stearothermophilus and GDP is polymerised smoothly in high yield. Copolymers were obtained from GDP and other nucleoside diphosphates in which the base ratios

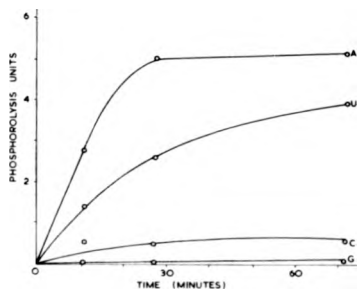


FIGURE 3  
PHOSPHOROLYSIS OF POLYNUCLEOTIDES UNDER STANDARD CONDITIONS  
BY T.AQUATICUS POLYNUCLEOTIDE PHOSPHORYLASE

Nucleic Acids Research

TABLE 1

PURIFICATION OF POLYNUCLEOTIDE PHOSPHORYLASES FROM THERMOPHILIC ORGANISMS

a) <u><i>B. stearothermophilus</i></u>			
Step	Specific Activity	Purification	Yield (%)
Cell lysate	1.2	1	100
35 - 55% ammonium sulphate	6.5	5	82
TEAE-cellulose	59	49	74
Sephadex A50	283	203	41
AcA 34 Ultrogel	603	500	41
b) <u><i>T. aquaticus</i></u>			
Step	Specific Activity	Purification	Yield (%)
Cell lysate	0.8	1	100
35 - 55% ammonium sulphate	4	5	57
TEAE-cellulose	25	31	21

TABLE 2

KINETIC PARAMETERS FOR *B. STEARTHERMOPHILUS* POLYNUCLEOTIDE PHOSPHORYLASE

a) <u>Polymerisation</u>		
Substrate	$K_m$ (mM)	$V_{max}$
ADP	2.5	100
UDP	2.9	30
CDP	2.9	27.5
GDP*	2.6	14
b) <u>Inhibition</u>		
Substrate	$K_i$ (mM)	
br <sup>3</sup> ADP*	0.21	
$\beta$ -azido ADP*	0.12	
pyrophosphate	0.04	
methylene diphosphate	0.28	

Polymerisation and inhibition studies were carried out under conditions as described in text.

\*Using  $Mn^{++}$  as metal cofactor, all others with  $Mg^{++}$ .

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TABLE 3

COPOLYMERISATION OF [ $^3\text{H}$ ]-GDP AND [ $^{14}\text{C}$ ]-UDP

GDP:UDP*	(G:U)
Input Ratio	Base Ratio of High Mol. Wt. Product
10	18
20	19
30	38
40	65

\*Specific radioactivities [ $^3\text{H}$ ]-GDP 24,000 cpm/mg, [ $^{14}\text{C}$ ]-UDP 26,000 cpm/mg. Reactions were carried out overnight under polymerisation assay conditions with final substrate concentrations of 10mM. Pancreatic RNase (50  $\mu\text{g}/\text{ml}$ ) and alkaline phosphatase (200  $\mu\text{g}/\text{ml}$ ) were then added and the mixture incubated at 37° for 3 hr. Deproteinisation with isocamyl alcohol: chloroform (5:2 v/v) followed by chromatography of the aqueous layer on Sephadex G25 with elution by 0.1% SDS and 0.2M KCl solution gave the products in the void volume.

correspond approximately to the input ratios of the component nucleotides. Unexplained anomalies between the input ratio and the subsequent composition of the polymer have been observed using *E. coli* polynucleotide phosphorylase (1), but we have not observed this with the *E. stearothermophilus* enzyme. Using a modification of the method of Tazawa et al,<sup>20</sup> we have prepared oligomers with a composition of 20 G: 1U, using [ $^3\text{H}$ ]-GDP and [ $^{14}\text{C}$ ]-UDP.

**DISCUSSION**

Purine nucleoside diphosphates in which the purine and the ribose are in the syn-conformation are poor substrates for polynucleotide phosphorylase<sup>18</sup>. As the temperature of a solution of such a purine nucleoside diphosphate is increased, rotation about the glycosidic bond should be facilitated and hence it may be that the rate of polymerisation by a thermostable polynucleotide phosphorylase

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would increase with temperature. We have found that 8-bromoadenosine and 8-bromoguanosine diphosphates, which are in the *syn*-conformation at room temperature<sup>18</sup>, are not substrates for polynucleotide phosphorylases from *B.stearothermophilus* or *T.aquaticus* at elevated temperatures. This lack of activity may be the result of a more rigid tertiary structure of the enzymes which might be necessary to avoid denaturation at the operating temperatures of the microorganisms in vivo. However the structural integrity of polynucleotide phosphorylases from thermophilic organisms makes them useful for physical studies. Proteolytic degradation products which contaminate most preparations from mesophilic sources have not been observed in the purification of the *B.stearothermophilus* enzymes, making structural studies considerably easier to accomplish. The gross amino acid composition of this enzyme does not differ markedly from those reported for *E.coli* and *M.luteus* enzymes, there being similar proportions of hydrophobic and hydrophilic amino acids. Our evidence for a tetrameric structure for *B.stearothermophilus* polynucleotide phosphorylase contrasts with recently published data on the quaternary structure of the *E.coli* enzyme<sup>21</sup>. Nevertheless we consider that *B.stearothermophilus* polynucleotide phosphorylase offers advantages for structural analysis which may well be of relevance to mesophilic enzyme.

We wish to thank the Science Research Council and G.D. Searle & Co. Ltd. High Wycombe, U.K. for financial support.

\*To whom reprint requests should be sent.

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- 2 Davidson, J.N. (1969) *The Biochemistry of the Nucleic Acids*, 6th edn, pp. 177-178, Methuen, London
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