Some studies on the use of polynucleotide phosphorylase in the synthesis of polynucleotides.

A Thesis submitted for the Degree of Doctor of Philosophy at the University of Warwick, by John C. Smith, November 1975.

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SUMMARY

The purification of polynucleotide phosphorylase (E.C.2.7.7.8) by affinity chromatography was investigated. Several affinity ligands were prepared and one of them, oligo (dT) Sepharose, was found to be an efficient adsorbent for the enzyme.

Polynucleotide phosphorylase, purified from Micrococcus luteus and Escherichia coli, was immobilized by covalent attachment to a number of insolubie supports. The properties of the immobilized enzyme were compared with those of the enzyme in free solution. It was found that while there was no significant change in the kinetic parameters of the enzyme, there was a significant increase in the stability of the immobilized derivatives. Studies were made of the use of the immobilized enzyme in the synthesis of polynucleotides.

Polynucleotide phosphorylase was isolated from Bacillus Stearothermophilus and was used to incorporate CDP residues into long oligonucleotide primers such as oligo(I) and t-RNA. Primers were isolated by chromatography on dihydroxyboryl Sepharose which ensured that the primers possessed an intact 3'-hydroxyl group. Although incorporation of CDP into the primer was achieved, the number of residues incorporated was low. However, the method could be used to label the 3'-terminus of a polynucleotide.

In an Appendix, the purification of an endonuclease from pig liver nuclei is described. The enzyme was used in the preparation of oligonucleotide primers.

ABBREVIATIONS

ATEE	N-acetyl-L-tyrosine ethyl ester.
BAEE	α-N-benzoyl-L-arginine ethyl ester.
BAPNA	α-N-benzoyl-DL-arginine-p-nitroanilide.
cl^5 CDP	5-chlorocytidine diphosphate.
ho^5 CDP	5-hydroxycytidine diphosphate.
DMF	Dimethyl formamide.
DMSO	Dimethyl sulphoxide.
DTT	Dithiothreitol.
MOPS	3-(N-Morpholino) propane sulphonic acid.
MPS	p-chloromercuriphenyl sulphonic acid.
TNBS	Trinitrobenzenesulphonic acid.

Polynucleotide phosphorylase

Polynucleotide phosphorylase (polynucleotide orthophosphate nucleotidyl transferase E.C.2.7.7.8) was first isolated from Azotobacter vinelandii in 1955 (Grunberg-Manago, Ortiz and Ochoa, 1955) and similar enzymes have been isolated from other bacterial sources, for example, E.coli (Littauer and Kornberg, 1957) and M.luteus (Beers, 1956) and from animal (Fitt and Coe, 1970) and plant (Brishammar and Juntti, 1974) sources.

The enzyme catalyses the reversible reaction,

$$_n NDP \rightleftharpoons (NMP)_n + Pi$$

The reaction is dependent on the presence of Magnesium (II) or Manganese (II) ions. The substrate specificity of the polymerization reaction is broad and if the concentration of nucleoside diphosphate is saturating, the rates of formation of poly(A), poly(C) and poly(I) are essentially the same. It was originally thought that the enzyme was absolutely specific for ribonucleoside diphosphates but recent work has shown that dADP can be incorporated into a primer by the M.luteus enzyme (Chou and Singer, 1971) and by the E.coli enzyme (Kaufmann and Littauer, 1969). For polymerization to take place, the nucleoside diphosphate must be capable of assuming the anti conformation (Kapuler, Monny and Michelson, 1970). The anti conformation normally present in nucleotides requires the close apposition of the 2'-proton of the ribofuranose ring and either the C-8 proton of the purines or the C-6 proton of the pyrimidines. The replacement of these ring protons by larger substituents such as bromine or methyl groups inhibits the assumption of the normal anti conformation resulting in inhibition of the polymerization reaction.

Polynucleotide phosphorylase has been purified to homogeneity from a number of sources and the physical properties of the enzymes are outlined in Table 1. It can be seen that the physical properties of the enzymes isolated from bacterial sources are similar. However, there have been several contradictory reports as to the value of the molecular weight

of the enzyme. A study of the E. coli enzyme (Portier etal., 1973) gave the following values for the molecular weight of the native enzyme : 220,000 (gel electrophoresis), 218,000 (sucrose gradient) and 216,000 $\pm$ 20,000 (sedimentation equilibrium). When the native enzyme was dissociated by SDS, two products were obtained with molecular weights of 92,000 and 46,000. Only the 92,000 molecular weight band could be renatured to give active enzyme. In a more recent paper, the following values have been proposed : native enzyme molecular weight 250,000, subunits molecular weight 85,000 (Portier, 1975). It was proposed that the native enzyme exists as a trimer and this was confirmed by the use of the cross-linking agent, dimethyl suberimidate. The enzyme from M. luteus is however, thought to exist as a tetramer (Klee, 1969).

Mechanism of action

(a) Polymerization

For many years, the study of the polymerization reaction was hampered by the lack of a pure enzyme, free of contaminating oligonucleotides. In particular, there was conflicting evidence as to the requirement for an oligoribonucleotide primer to initiate polymerization. The enzymes from E. coli and M. luteus have been purified to homogeneity and it has been shown that both enzymes are capable of de novo polymerization in the absence of oligonucleotide primer (Godefroy, Cohn and Grunberg-Manago, 1970; Moses and Singer, 1970).

The role of oligonucleotides in the polymerization reaction catalysed by the E. coli enzyme has been studied in detail (Godefroy, Cohn and Grunberg-Manago, 1970). De novo polymerization catalysed by the pure enzyme shows a lag phase which can be overcome by the addition of 3'-hydroxyl oligonucleotides. It was proposed that there were multiple binding sites on the enzyme. In the case of de novo polymerization, the enzyme would form oligo and polynucleotides which would bind strongly at the binding sites and maintain the enzyme in the most suitable conformation for polymerization. This would explain the autocatalytic behaviour of the enzyme. When oligonucleotides are added to the enzyme, the enzyme

TABLE 1

Physical properties of polynucleotide phosphorylase

Source	Molecular weight	Subunits	Reference
E. Coli	220,000	92,000, 48,000	Portier et al, (1973)
	250,000	85,000	Portier, (1975)
M. luteus I x	270,000	100,000	Letendre and Singer, (1975)
T xx	230,000	71,000	
Streptomyces aureofacieus	210,000	100,000	Simuth, Zelinka and Polek (1975)
Tobacco plant	150,000		Brishammar and Juntti, (1974)

* Form I primer independent form

** Form T primer dependent form obtained by tryptic digest.

assumes the correct conformation and does not exhibit a lag phase. A model of the proposed polymerization scheme is shown in Figure 1.

In the case of the M. luteus enzyme most studies have concentrated on the interconversion of the primer independent form of the enzyme (PNPase-I) and the primer dependent form (PNPase-T). PNPase-I has a molecular weight of 270,000 and exhibits several bands of activity on gel electrophoresis. PNPase-T is obtained by limited tryptic digestion of PNPase-I, has a molecular weight of 230,000 and exhibits a single band of activity on gel electrophoresis(Klee, 1969; Letendre and Singer, 1975). The amino acid composition of the two forms of the enzyme have been studied and it has been found that PNPase-I contains 8-10 cysteine residues while PNPase-T contains 4-5 cysteine residues. The loss of lysine, arginine, leucine and isoleucine is greater than would be expected from a random digestion by trypsin (Letendre and Singer, 1975).

If PNPase-T is incubated in the presence of 2-mercaptoethanol or dithiothreitol, the enzyme becomes primer independent but will revert to primer dependence if treated with sulphydryl inhibitors such as N-ethylmaleimide or p-chloromercuriphenyl sulphonic acid. The effect of p-chloromercuriphenyl sulphonic acid is reversed by sulphydryl reagents but the effect of N-ethylmaleimide is irreversible (Klee and Singer, 1968b) (Figure 2). PNPase-I reacts with N-ethylmaleimide although under normal polymerization conditions less than 1 mole of N-ethylmaleimide reacts per mole of enzyme. If the reaction is carried out in the presence of a substrate, such as ADP, the extent of reaction with N-ethylmaleimide is markedly reduced (Letendre and Singer, 1974). If the enzyme is incubated in the presence of 1.0 M guanidinium chloride, an additional sulphydryl group reacts with N-ethylmaleimide. However, if the enzyme is reduced in the presence of guanidinium chloride, a total of 3 - 4 sulphydryl groups will react. The enzyme is now primer dependent but shows the same properties on gel electrophoresis as PNPase-I. The presence of poly(A) or $(Ap)_4A$ cyclic phosphate prevents the loss of primer independency but not the reaction with N-ethylmaleimide. The fact that poly(A) protects the enzyme from loss

FIGURE 1

Polymerization of nucleoside diphosphates by E. Coli polynucleotide phosphorylase.

Godefroy, Cohn and Grunberg-Manago, 1970

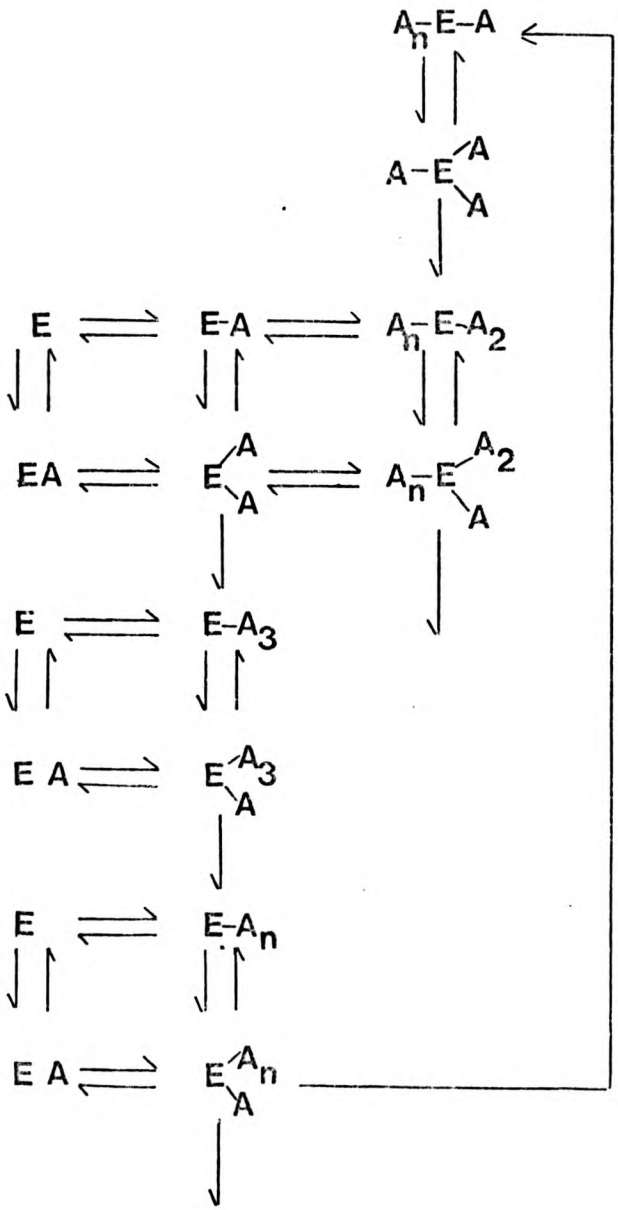
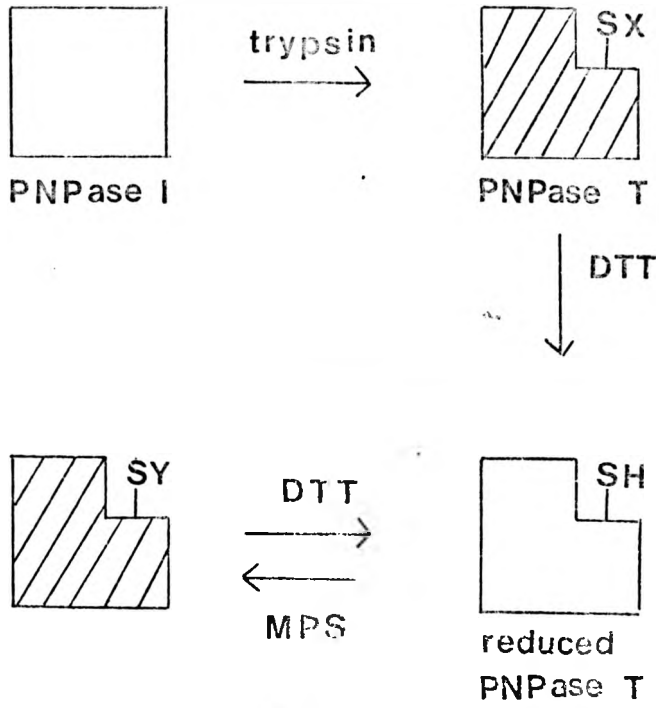

A_n-E-A
A-E(A)A
E
E-A
A_n-E-A_2
EA
E(A)A
A_n-E(A_2)A
E
E-A_3
E A
E(A_3)A
E
E-A_n
E A
E(A_n)A

FIGURE 2

Interconversion of primer independent and primer dependent forms of M. luteus polynucleotide phosphorylase.

Klee and Singer, 1968b

Cross-hatching indicates primer dependency.


trypsin
SX
PNPase I
PNPase T
DTT
SY
DTT
SH
MPS
reduced
PNPase T

of de novo polymerization activity even though reaction with NEM has taken place, while ADP under the same conditions does not, suggests that poly(A) is maintaining a conformation suitable for de novo synthesis possibly through multiple site interactions.

(b) Phosphorolysis

There are two possible mechanisms for the degradation of a polymer into monomeric units by an enzyme. In a processive mechanism the enzyme binds to the polymer and is not released until the polymer has been completely degraded, while in a random mechanism, the enzyme polymer complex is constantly dissociating and reassociating. The M. luteus enzyme has been shown to degrade polynucleotides in a processive manner (Klee and Singer, 1968a) although short oligonucleotides are degraded in a random manner. Similar results were obtained by Thang et al (1967) in a study of the phosphorolysis of t-RNA by the E. coli enzyme. Experiments have been carried out with substrates of various chain length in order to determine the point at which the mechanism changes from processive to random. With the E. coli enzyme, the values of Km for various oligo(A) substrates decreased with increasing chain length while the Km of polymer substrates was several orders of magnitude lower (Godefroy, 1970). There was a sharp transition in the values of Km for substrates with chain lengths between 10 and 40. A model was proposed in which there are two separate binding sites for the polymer substrate. Site I binds the substrate at the terminal 3'-hydroxyl and phosphorolysis takes place at site I. The remainder of the polymer is tightly bound to Site II and remains bound to the enzyme until it is not long enough to bind simultaneously at Sites I and II.

A detailed kinetic analysis of the phosphorolysis of oligonucleotides and polynucleotides by the M. luteus enzyme has been carried out in order to establish the mechanism of this enzyme (Chou and Singer, 1970, a, b; Chou, Singer and McPhie, 1975). When $(Ap)_4A$ and Pi were used as substrates, linear double reciprocal plots of initial velocity against substrate were obtained with both substrates. The affinity of one substrate for the enzyme was unaffected by the presence of the other substrate.

Oligonucleotides with 3' phosphate groups were found to be competitive inhibitors with respect to oligonucleotides and noncompetitive with respect to inorganic phosphate. Two product analogues, dADP and adenosine 5' methylene diphosphonate, are competitive inhibitors against inorganic phosphate and noncompetitive inhibitors against oligonucleotide. Similar results were obtained with the primer independent and primer dependent forms of the enzyme. The data were consistent with a rapid equilibrium random Bi-Bi mechanism (Cleland, 1963). The mechanism is shown in Figure 3. A similar mechanism has been proposed for a polysaccharide phosphorylase from E. coli (Chao, Johnson and Graves, 1969).

Further studies have been carried out on the phosphorolysis of oligonucleotides by polynucleotide phosphorylase from M. luteus in order to determine the effect of chain length and base composition (Chou and Singer, 1970 b). The phosphorolysis of a series of oligo(A) and oligo(U) oligomers was studied and it was found that the Km decreased with increasing chain length and reached a minimum value when the oligonucleotide contained 5 phosphate residues. If the chain length is less than 5 the Km is dependent on the base residues since the Km of oligo(U) is consistently higher than the Km for oligo(A) substrates. If the mechanism is rapid equilibrium random Bi-Bi, the Km represents the dissociation constant for oligonucleotide. This is confirmed by the observation that the Ki of phosphosphorylated oligonucleotides (which act as competitive inhibitors) is similar to the Km of oligonucleotide substrates of the same chain length. On the basis of these studies, Chou and Singer were able to propose a model of the binding site for oligonucleotides in polynucleotide phosphorylase (Figure 3). There are multiple binding sites and each subsite contains a nucleoside binding region (N) and a phosphate binding region (P). Subsite I will bind the terminal nucleotide of the oligonucleotide while subsite Pi binds inorganic phosphate. These two sites are independent of each other (Chou and Singer, 1970 a). In the polymerization reaction, the nucleoside monophosphate portion of the substrate will interact with subsite I while the β phosphate reacts with subsite Pi. The observation that the Km of

FIGURE 3

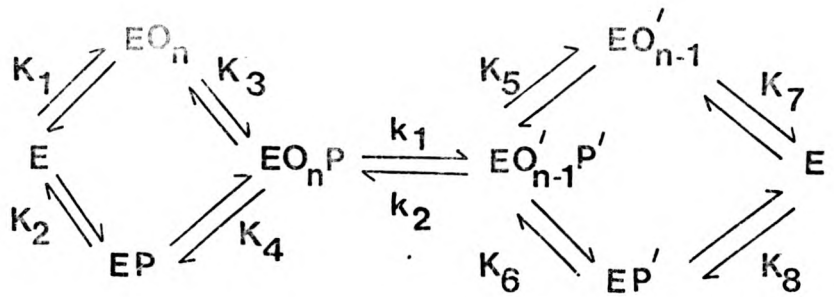
A Rapid equilibrium random BiBi mechanism

B Scheme of active site of M. luteus polynucleotide phosphorylase

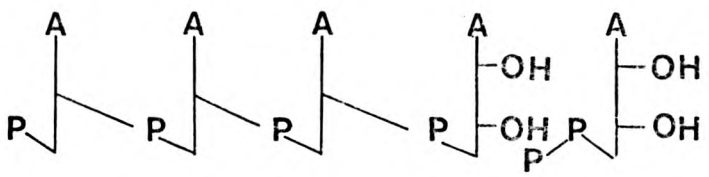
1. polymerization
2. phosphorolysis

Chou and Singer, 1970 a, b

A


EO$_n$
K$_1$
K$_3$
E
EO$_n$P
K$_2$
K$_4$
EP
k$_1$
k$_2$
EO$'_{n-1}$
K$_5$
K$_7$
EO$'_{n-1}$P$'$
E
K$_6$
K$_8$
EP$'$

B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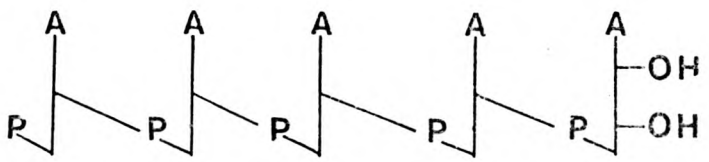
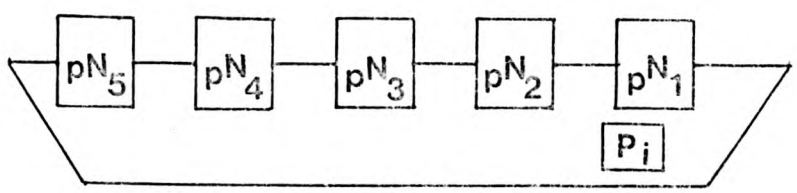
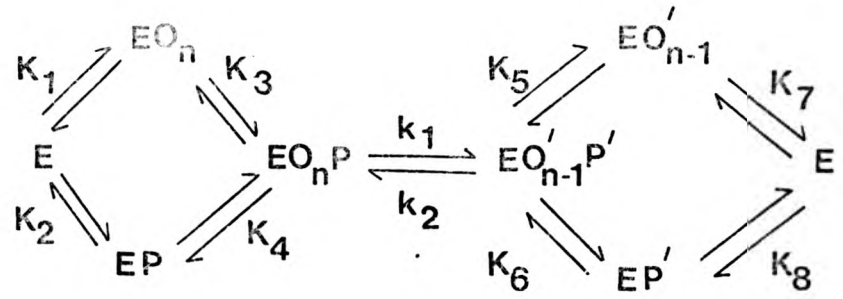
P
OH
1

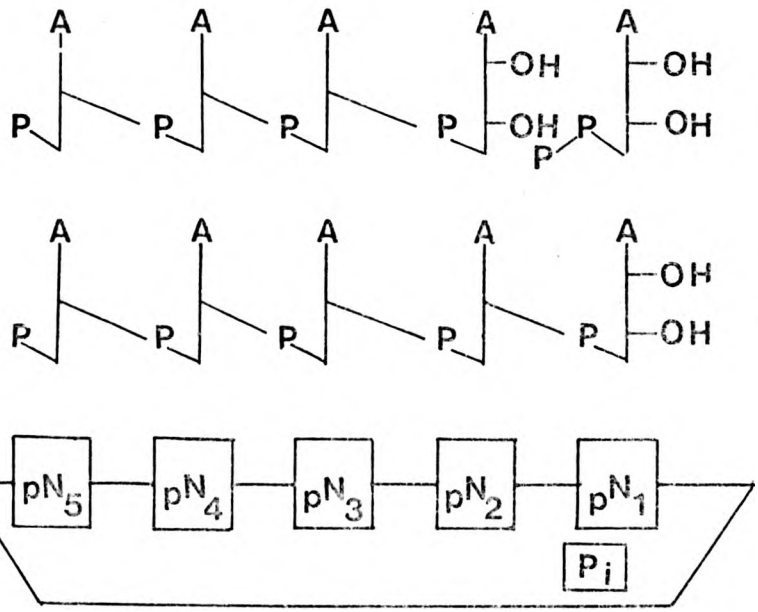

A
P
OH
2


pN$_5$
pN$_4$
pN$_3$
pN$_2$
pN$_1$
P$_i$

A


EO$_n$
K$_1$
K$_3$
E
EO$_n$P
K$_2$
K$_4$
EP
k$_1$
k$_2$
EO$'_{n-1}$
K$_5$
K$_7$
EO$'_{n-1}$P$'$
E
K$_6$
EP$'$
K$_8$

B


A
P
OH
1
2
pN$_5$
pN$_4$
pN$_3$
pN$_2$
pN$_1$
P$_i$

an oligonucleotide decreases with increasing chain length until there are 5 phosphate residues, suggests that there are at least four more subsites. The base-enzyme interaction plays a critical role in the binding of substrate as can be seen from the higher Km values of uridine oligonucleotides compared with adenosine oligonucleotides. This is most marked in the case of trinucleotides since ApApA is a good substrate while UpUpU is not phosphorolyzed.

The kinetic mechanism of polynucleotide phosphorolysis has recently been studied in detail (Chou, Singer and McPhie, 1975). Linear double reciprocal plots were obtained with inorganic phosphate or poly(A) as substrate and the affinity of either substrate for the enzyme was independent of the presence of the other. dADP was a competitive inhibitor with respect to inorganic phosphate and poly(A). $(Ap)_5A$ cyclic phospnate was competitive with respect to poly(A) and noncompetitive with respect to inorganic phosphate. Similar results were obtained with primer independent and primer-dependent forms of the enzyme, although the values of the kinetic constants differed. On the basis of these data a rapid equilibrium random Bi-Bi mechanism was proposed. This is the same mechanism as that proposed for oligonucleotide phosphorolysis but there are several important differences. dADP is a competitive inhibitor with respect to polynucleotide but is noncompetitive with respect to oligonucleotide and the Ki values with dADP as a competive inhibitor with respect to inorganic phosphate and polynucleotide are not equal. The Ki value for poly(A) as an inhibitor of oligonucleotide phosphorolysis is very much lower than Km for poly(A) phosphorolysis while the Ki and Km of oligonucleotide inhibitors and substrates are similar.

It is proposed that although phosphorolysis of a polynucleotide proceeds with a rapid equilibrium random Bi-Bi mechanism, the longer chain length allows the polymer to bind to the enzyme at a second site. When one unit is removed, the polymer does not dissociate from the enzyme and is degraded in a processive manner. This model for the M. luteus enzyme is similar to that proposed for the enzyme from E. coli (Godefroy, 1970).

Physiological Role

The function of polynucleotide phosphorylase in vivo could be the synthesis or degradation of polynucleotides. However, the comparatively low affinity of the enzyme for nucleoside diphosphates would seem to make it unlikely that polymerization is the significant in vivo function.

The role of polynucleotide phosphorylase in E. coli has been studied using mutants with modified polynucleotide phosphorylase activities. Three strains, Q7, Q13 and Q27 were isolated from mutagenized cultures and these strains were then transduced by phage P1 into genetically characterized strains giving strains which only differed in the polynucleotide phosphorylase locus (Reiner, 1969a). The enzyme from strain Q7 showed very low activity, the enzyme from strain Q13 had low activity which could be increased in the presence of manganous ions while the enzyme from strain Q27 was thermolabile and was inactivated at 44° if the cells were prevented from growing (Reiner, 1969b).

The growth of mutants with low levels of polynucleotide phosphorylase has been studied. All mutants with low levels of the enzyme grew slowly at 45° and when revertants were isolated which could grow at 45°, they were all found to have increased levels of polynucleotide phosphorylase (Krishna and Apirion, 1973). Similarly, there was a good correlation between the constitutive levels of polynucleotide phosphorylase and the inducible level of tryptophanase. The ability of a culture to grow to a given cell density was also found to be related to the level of polynucleotide phosphorylase in the cells. Further studies on the stability of m-RNA molecules during exponential growth suggested that polynucleotide phosphorylase was not involved in their degradation (Krishna, Rosen and Apirion, 1973). Two strains of E. coli, PR 27 (pnp^-) and PR100 (pnp^+) were grown at 49° and the half lives of total pulse labelled RNA and m-RNA were studied. The decay of RNA was much faster in PR27 than PR100, similarly the half life of β-galactosidase m-RNA was much shorter in PR27 and the ability to synthesize peptides was diminished in the pnp^- mutant. These

results suggest that during exponential growth, high levels of polynucleotide phosphorylase stabilize m-RNA molecules.

However, under some conditions polynucleotide phosphorylase can function as a degradative enzyme. When cultures of E. coli are grown under conditions of carbon starvation, ribosomal RNA and t-RNA are rapidly degraded by ribonucleases and polynucleotide phosphorylase (Kaplan and Apirion, 1974). Ribosomal subunits are degraded by an endonuclease to RNA molecules about 4S in size. The 4S molecules are then degraded by polynucleotide phosphorylase to 5' nucleoside diphosphates. When mutants defective in RNA decay are isolated, they are found to be defective in polynucleotide phosphorylase (Kinscherf, Fon Lee and Apirion, 1974). If polynucleotide phosphorylase does function by degrading RNA, this would be advantageous to the cell as the degradation products are 5' diphosphates. This results in a conservation of energy which would be lost if the RNA was degraded to 5' monophosphates.

In conclusion, the exact role of the enzyme cannot be stated but the available evidence suggests that it can function in the synthesis or degradation of RNA molecules depending on the environmental conditions.

Applications

One of the first uses of the enzyme was in the production of polynucleotides for physical studies and for evaluation as interferon inducers. More recently, attention has been concentrated on the use of the primer dependent enzyme in the synthesis of defined sequence oligonucleotides.

Thach and Doty (1965a, b) have studied the synthesis of defined sequence oligonucleotides in great detail. In their studies with the enzyme from M. luteus, it was found that the ionic strength had a critical effect on the reaction. There was an inverse relationship between the ionic strength and the chain length of the oligonucleotide formed. The block oligonucleotides formed can be purified by chromatography and used as a primer for further additions. The yields of the reaction can be improved by incorporating a phosphate removal system, consisting of nucleoside phosphorylase and nicotinamide riboside, into the reaction mixture

(Sninsky, Bennett and Gilham, 1974). Deoxynucleotides can be incorporated into ribonucleotide primers, for example, two thymidine residues can be incorporated into $(Ap)_5A$ (Feix, 1972) and dAMP residues can be incorporated into ApA or ApU (Kaufmann and Littauer, 1969). During the purification of E. coli polynucleotide phosphorylase, an enzyme capable of polymerizing deoxynucleoside diphosphates is co-purified. Since the two activities are inseparable, it is thought that the ability to polymerize deoxynucleoside diphosphates is a property of polynucleotide phosphorylase (Hsieh, 1971; Gillam and Smith, 1974). E. coli polynucleotide phosphorylase has since been used to prepare oligodeoxynucleotides containing up to nine residues (Gillam et al., 1974).

Polynucleotide phosphorylase has been immobilized on cellulose, producing a stable derivative of the enzyme which can be reused many times in the synthesis of polynucleotides (Hoffman et al., 1970). The major aim of the present project was to prepare immobilized derivatives of the enzyme using other support materials and to study the properties of these derivatives. The purification of the enzyme by affinity chromatography was also investigated. The properties of immobilized enzymes and the technique of affinity chromatography will be reviewed in the following section.

Immobilized Enzymes

Enzymes have great potential for industrial use because of their specificity and the wide number of complex reactions which they can catalyse. However, several factors mitigate against the use of purified enzymes on a large scale. The most important of these are the high cost of isolation and the difficulty of recovering an enzyme from the reaction mixture. In the past, this has meant that many industrial reactions have been carried out by intact micro-organisms.

In recent years, two major developments have improved the prospects for the use of enzymes on an industrial scale. The first of these is the improvement of enzyme purification techniques through the scaling up of existing methods and the use of new techniques such as affinity chromatography. The second development has been the preparation of immobilized enzyme derivatives which retain their specificity and catalytic activity and can be used repeatedly (Silman and Katchalski, 1966; Melrose, 1971).

Nelson and Griffin (1916) prepared the first immobilized enzyme when they adsorbed invertase onto charcoal and found that the enzyme retained activity. Following the preparation of water insoluble antigens and antibodies, the field of immobilized enzyme technology has developed rapidly.

There are four main methods for the preparation of immobilized enzymes.

1. Adsorption onto carriers

This is not a very reliable method as it is nonspecific and protein can be lost through changes in pH, ionic strength or temperature. The method has been used to prepare derivatives of aminoacylase adsorbed onto DEAE-cellulose (Sato et al., 1971).

2. Entrapment in gels or microcapsules

This method has the advantage that covalent bond formation is not required and the method can therefore be applied to any enzyme.

However, there are several disadvantages. Leakage of enzyme is difficult to avoid, catalysis is limited by diffusion of substrate into the gel and free radicals, generated during the polymerization of the gel, may affect the activity of the enzyme. The technique has been used to prepare microcapsules containing L-asparaginase (Chang, 1972)

3. Covalent cross-linking of the protein by bifunctional reagents

The most common type of derivative prepared is one in which only one enzyme is cross-linked, for example trypsin reacts with glutaraldehyde to form an insoluble, polymeric derivative which retains activity (Habeeb, 1967). Other derivatives have been prepared in which the enzyme is crosslinked to an antibody (Avrameas, 1969) or to another enzyme (Mattiasson, Johansson and Mosbach, 1974). A disadvantage of the method is that only a small percentage of the enzyme molecules are available for reaction with substrate. The activity of the preparations can be improved by adsorbing the enzyme onto a surface and then cross-linking the enzyme molecules (Haynes and Walsh, 1969).

4. Covalent binding of proteins to an insoluble matrix via functional groups on the enzymes

This is the most widely used and satisfactory method of preparing immobilized enzymes. In general, support materials can be divided into two classes - organic and inorganic. Typical examples of organic supports are cellulose and other polysaccharides, polyacrylamide, nylon and polystyrene. Cellulose derivatives are readily available and can be relatively easily converted to activated forms. For example, carboxymethyl cellulose can be converted to an acid azide which will then react with proteins at alkaline pH (Mitz and Summaria, 1961). Another method of activating cellulose derivatives is to react them with a triazine (Kay and Lilly, 1970). The polysaccharides, Sephadex and Sepharose are very useful for enzyme immobilization as they are readily activated by cyanogen bromide (Axen, Porath and Ernback, 1967) and have low levels of impurities which could lead to non-specific adsorption of protein. The use of polyacrylamide as a support material has been described by Inman and Dintzis (1969) and the use of nylon and polystyrene has been developed by Hornby and co-workers (Inman and Hornby, 1972; Filippusson and

Hornby, 1970). Nylon has been found to be the more suitable material as the hydrophobic environment of polystyrene can lead to enzyme inactivation.

Inorganic support materials offer the advantage of resistance to microbial attack and since the materials are rigid, they can be used in packed beds at high flow rates. The technique of attaching enzymes to inorganic supports has been developed by Weetall (1969, 1970) and can be applied to a variety of materials such as porous glass and nickel oxide.

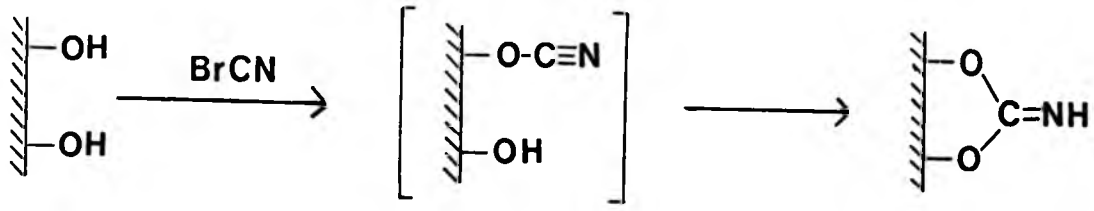
In the present work, three main techniques were used and will now be described in greater detail. They were, cyanogen bromide activation of polysaccharides, triazine activation of DEAE-cellulose and coupling of the enzyme to aminoalkyl derivatives of porous glass and celite.

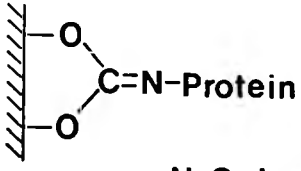
Cyanogen bromide activated polysaccharides are widely used in the preparation of immobilized enzymes and in the synthesis of materials for affinity chromatography. The advantages of the method are its simplicity, mild reaction conditions and the stability of the products. Studies with activated polymers and model compounds have led to the proposal of a reaction scheme (Figure 4) (Axen and Ernback, 1971; Kagedal and Akerstrom, 1971). It was concluded that activation of the polymer leads to the formation of cyclic imidocarbonates and carbamates. Of these, the imidocarbonate is the dominant species and reacts most strongly with nucleophilic groups in proteins, such as the ε amino group of lysine. The active species formed during cyanogen bromide activation have been characterized by studying the behaviour of an activated soluble dextran on isoelectric focussing (Svensson, 1973). The polymer was less positively charged, indicating that the imidocarbonate was the major product. This finding was in agreement with the assignments based on infra-red studies of model compounds (Bartling et al., 1972).

Cellulose and Sephadex should be more readily activated by cyanogen bromide than agarose as these polymers are made up of β 1---) 4 linked glucose residues (Fig. 5) (Porath, 1968). The 2, 3 hydroxyl groups of the glucose based polymers are diequatorial and should form the cyclic

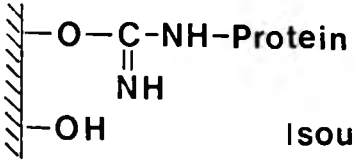
FIGURE 4

Activation of polysaccharides by cyanogen bromide and subsequent reaction of the activated matrix with proteins. (Axen and Ernback, 1971)





N-Substituted Imidocarbonate

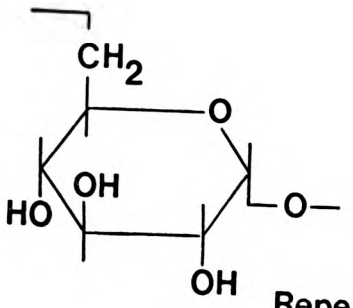


Isourea Derivative

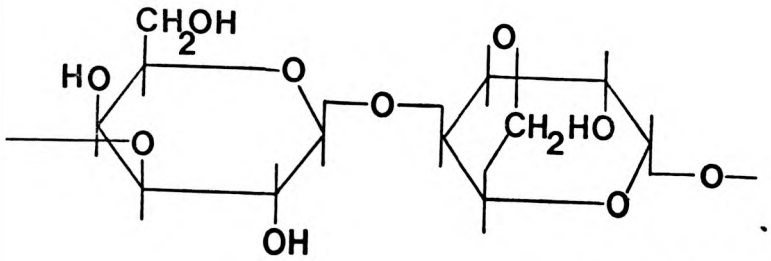
FIGURE 5

Structures of Sephadex and Sepharose.

(Porath, 1968)



Repeating unit in Sephadex



Repeating unit in Agarose

imidocarbonate more readily than the agarose derivatives. Model studies on the coupling of glycyl-leucine to activated polymers have shown that Sephadex can bind up to three times the amount of glycyl-leucine coupled to agarose which had been activated under similar conditions (Axen and Ernback, 1971).

Until recently, the bond between activated agarose and a protein was presumed to be stable (Cuatrecasas, 1970) but recent experiments with insulin covalently bound to Sepharose have indicated that this might not be the case. When insulin-Sepharose was incubated with BSA, an active soluble form of insulin was released (Oka and Topper, 1974). Further studies indicated that nucleophilic attack by amines could release N_1 N_2-disubstituted guanidines (Wilchek, Oka and Topper, 1975). This could cause significant losses if the immobilized enzyme was used in a continuous process.

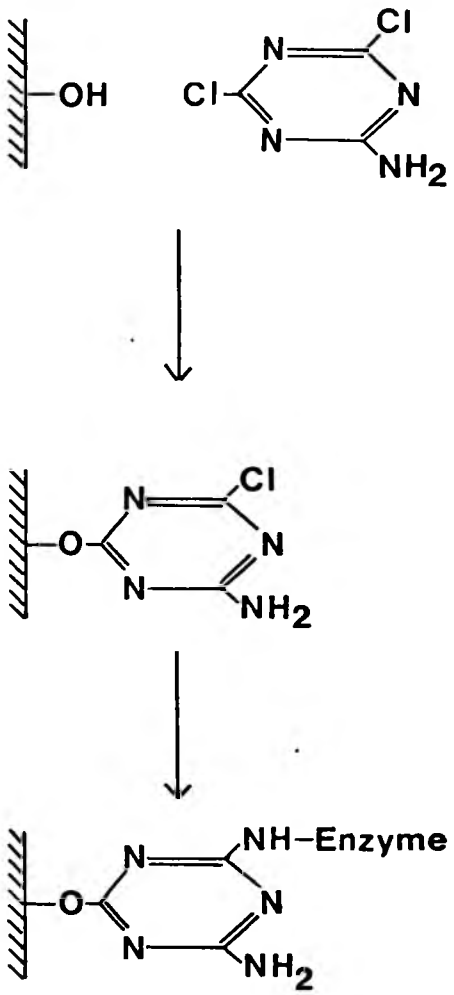
Agarose gels become crosslinked during activation and become more rigid. This is useful if the immobilized enzyme is going to be used in a continuous process as it minimizes enzyme loss. A new process for activating agarose gels has recently been described which leads to a highly crosslinked gel containing reactive oxirane groups which can react with amine and hydroxyl groups (Sundberg and Porath, 1974). The crosslinked polymer is stable at high temperatures and could be useful for the immobilization of enzymes from thermophilic bacteria.

The second class of derivatives was prepared by the reaction of the enzyme with cellulose derivatives which had been activated by sym-triazine (Fig. 6) (Kay and Lilly, 1970; Wilson, Kay and Lilly, 1968). It is possible to choose the net electrostatic charge of these derivatives leading to higher protein loadings. Since reaction proceeds through the hydroxyl groups of the matrix, ion exchange celluloses such as CM - and DEAE - cellulose can be used as support materials. Cyanuric chloride is too reactive and its use results in an aggregate of crosslinked material. The most commonly used reagent is 2-amino-4, 6-dichloro-sym-triazine. The activated matrix reacts predominantly with lysine groups on the surface of the enzyme (Kay and Crook, 1967).

.FIGURE 6

Reaction of polysaccharides with 2-amino - 4,6 - dichloro-s-triazine and subsequent reaction of the activated matrix with proteins.

(Kay and Lilly, 1970).


OH
Cl
N
Cl
N
N
NH2
O
NH–Enzyme

The third group of enzyme derivatives were based on the inorganic materials, porous glass and celite-560. Porous glass beads are manufactured from 96% silica and can be purchased in well defined pore sizes. Celite-560 was used as an inexpensive alternative to the defined pore beads and is a form of silica containing metal oxide impurities. The first stage in the process is the formation of an alkylamine derivative which can then be reacted with enzymes in a number of ways. A diazonium derivative can be formed which will react with tyrosine residues, the amino group can be converted to an isothiocyanate which can then be reacted with lysine and arginine residues. The amine group can be condensed with carboxyl groups by means of a water soluble carbodiimide. Perhaps the simplest method is to react the alkyl amine glass with glutaraldehyde, the activated matrix will then bind to lysine residues on the enzyme through Schiff's bases (Robinson, Dunnill and Lilly, 1971) (Fig. 7).

A disadvantage of porous glass is its fragility in a stirred reactor which can be overcome by the use of zirconium oxide coated glass beads (Weetall and Havewala, 1972). Several other inorganic materials have been proposed as support materials, for example, nickel oxide (Weetall and Hersh, 1970) and alumina silica (Herring, Laurence and Kitrell, 1972). Possible developments include the use of magnetic support materials which could be readily removed from the reaction mixture (van Leemputter and Horisberger, 1974).

Properties of immobilized enzymes

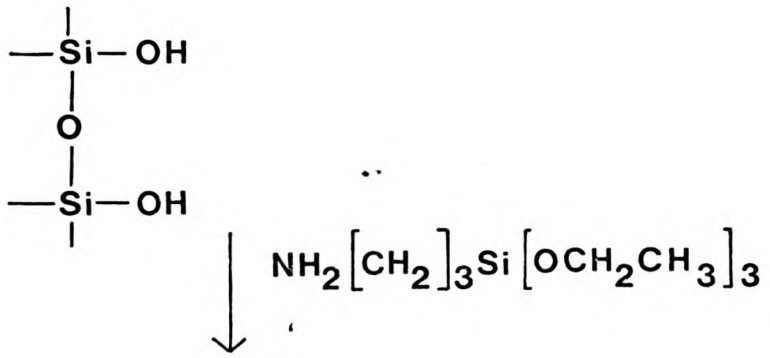
Stability

One of the most desirable features in an industrial catalyst is stability, both in use and in storage. In most cases, immobilized enzymes have shown increased stability on prolonged storage and an increased resistance to denaturants and extremes of temperature. For example, chymotrypsin-agarose conjugates can be stored at 4° in buffer for up to 18 months (Axen and Ernback, 1971) and immobilized β-galactosidase is stable over a period of years (Lilly, 1971). Furthermore, when urease is attached to nylon, its thermal stability and storage properties are

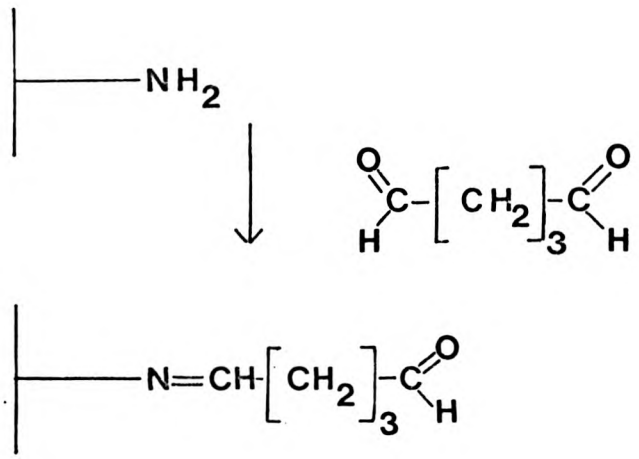
FIGURE 7

Preparation of alkylamine derivatives of glass and activation with glutaraldehyde.

A


Si—OH
O
Si—OH
NH2[CH2]3Si[OCH2CH3]3
Si—O—Si[CH2]3NH2
O
O
Si—O—Si[CH2]3NH2

B


NH2
O
C
H
[CH2]3
C
O
H
N=CH[CH2]3C
O
H

enhanced (Sundaram and Hornby, 1970). Lactate dehydrogenase attached to DEAE-cellulose also shows an increase in thermal stability (Wilson, Kay and Lilly, 1968).

The resistance of immobilized trypsin to urea denaturation has been studied in detail (Gabel, 1973). When trypsin is attached to Sephadex, it is resistant to denaturation by 8 M urea. However, trypsin-agarose conjugates are not resistant to the same concentrations of urea. Derivatives of chymotrypsin-Sephadex were not resistant to denaturation by urea (Gabel et al., 1970). The fluorescence spectrum of the trypsin-Sephadex conjugate was unaltered (Gabel, Steinberg and Katchalski, 1971). The resistance to urea denaturation could be due to a number of reasons. The presence of carbohydrate residues could reduce the likelihood of the protein unfolding, modification of the amino groups could lead to an alteration in the denaturation mechanism and finally, the cross-linking between the protein and matrix could prevent the conformational changes which take place during denaturation.

Not all enzymes exhibit enhanced stability when immobilized, peroxidase bound to CM-cellulose is less stable than the free enzyme (Weliky, Brown and Dale, 1969) and the stability of derivatives of trypsin and maleicanhydride-ethylene copolymers depends on the concentration of enzyme coupled (Weetall, 1970).

Kinetics

There are four main reasons for a change in the behaviour of an immobilized enzyme when compared with that of the enzyme in free solution (Sundaram, Tweedale and Laidler, 1970; Kobayashi and Laidler, 1973).

1. There may have been a conformational change in the enzyme as a result of its covalent attachment to the matrix.

2. The enzyme-substrate interactions occur in a different environment.

3. There will be partioning of the substrate in free solution and in the microenvironment of the matrix. These effects will be especially pronounced if the matrix and substrate are electrically charged.

4. In free solution enzyme reactions are not usually diffusion-controlled, but the rate of reaction of an immobilized enzyme may be limited by diffusional or mass-transfer effects.

Little is known of the effect of covalent attachment of an enzyme to a matrix. Only 6% of the protein in an immobilized glucose oxidase derivative was found to be catalytically active (Weibel and Bright, 1971). This 6% of protein was bound on the surface of the matrix while the inactive protein was bound within the porous network of the support. The difficulty of distinguishing active from inactive protein has made it hard to calculate meaningful values of the catalytic constant, kcat. For example, the apparent kcat of a CM-cellulose bromelain derivative was found to be 52% of the value of the free enzyme when calculated on the basis of protein content. When the value was calculated on the basis of active thiol groups, the apparent kcat rose to 77% of the free enzyme value (Wharton, Crook and Brocklehurst, 1968a). More recently, an active site titration method for the determination of active trypsin has been developed and it was found that only 25% of an immobilized trypsin derivative was active. (Ford, Chambers and Cohen, 1973).

Effect of charged matrices on the kinetic constants of immobilized enzymes

Several groups of workers have shown that the interaction between the matrix and a charged substrate has a critical effect on the apparent values of kinetic constants for an immobilized enzyme. For example, ATP creatine phosphotransferase attached to CM-cellulose shows a tenfold increase in the apparent value of Km with respect to ATP which is negatively charged (Hornby, Lilly and Crook, 1967) while a CM-cellulose ficin derivative shows a tenfold decrease in apparent Km where the substrate, BAEE, is positively charged (Hornby, Lilly and Crook, 1966). There is little apparent change in the Km for enzymes whose substrates are uncharged. This effect is caused by the interaction of substrate and matrix. If they have opposite charge, there will be an increase in the local concentration of substrate in the microenvironment of the enzyme, the effect is overcome

at high ionic strength (Wharton, Crook and Brocklehurst, 1968b). The effect is not restricted to immobilized enzymes as the apparent Km of soluble polycationic derivatives of chymotrypsin is higher than the value observed with the unmodified enzyme with ester substrates. There was no significant change in the values of Km for the polyanionic derivatives of this enzyme (Goldstein, 1972).

Effect of immobilization on specificity and kinetic pathways of enzymes

None of the immobilized enzymes studied to date have shown a real change in substrate specificity or kinetic mechanism. One enzyme which has been studied in detail is D-3-hydroxy butyrate dehydrogenase (Preuveneers et al., 1973) and there was found to be very little difference in the values of the rate constants observed when the enzyme was in free solution and when it was covalently bound to DEAE-cellulose. Kinetic analysis indicated that the reactions followed by free and immobilized enzyme followed the same pathway.

The most marked effects on immobilized enzymes are observed with high molecular weight substrates. The hydrolysis of casein by CM-cellulose ficin is very much slower than the corresponding hydrolysis of low molecular weight substrates (Hornby, Lilly and Crook, 1966). This is probably caused by steric hindrance which can be overcome by the use of a soluble matrix such as dextran (Wykes, Dunnill and Lilly, 1971). Steric hindrance can give rise to an apparent change in specificity in favour of low molecular weight substrates. For example, trypsin cleaved fifteen peptide bonds in pepsinogen whereas an immobilized derivative only cleaved ten peptide bonds (Ong, Tsang and Perlmann, 1966).

Effect of diffusion on immobilized enzymes

Several studies of immobilized enzymes have shown that diffusion can influence the rate of reaction. For example, the apparent Km for the hydrolysis of BAEE catalysed by packed beds of CM-cellulose ficin increased at low flow rates (Lilly, Hornby and Crook, 1966). The apparent value of Km decreased at higher flow rates, finally reaching the same value as that obtained in stirred suspension. The results were

consistent with the presence of a diffusion layer around each particle of immobilized enzyme. The rate of transfer of substrate through the diffusion layer will be inversely proportional to the thickness of this layer which is itself inversely related to the flow rate. At lower flow rates, there will therefore be a difference between the concentration of substrate in the reaction medium and the concentration of substrate in the micro-environment of the enzyme.

These results have been extended by Taylor and Swaisgood(1972) who attached trypsin to polystyrene beads through spacer arms of glycine residues. The observed values of kcat were found to be independent of flow rate and the chain length of the spacer. The apparent Km was dependent on both parameters. A decrease in the thickness of the diffusion layer occurred upon doubling theflow rate and when the spacer consisted of four glycine residues, the decrease was 55 microns. In the case of a ten residue spacer, the decrease was 22 microns. An increase in apparent Km with increasing spacer length occurred suggesting that factors other than diffusion also influence the observed value of Km. For example, there could have been a change in the binding constant for substrate caused by the hydrophobic environment of the polystyrene matrix.

The kinetics of immobilized chymotrypsin have been studied in a stirred batch reactor (Lilly and Sharp, 1968). The substrate used, ATEE, was uncharged and the reaction was not influenced by substrate or product inhibition, allowing interpretation of the results solely in terms of diffusion effects. They found that the rate of stirring could influence the values of Km and at high stirring rates, the Km reached a constant value.

The effects described above have been due to external film diffusion. If an enzyme is immobilized on a porous support material, the majority of the enzyme will be located inside the pores of the matrix. Substrate molecules will not only have to diffuse through the external film layer but will also have to travel through the pores to reach the active site of the enzyme. This pore diffusion represents a further limitation on the activity of immobilized enzymes.

The problem has been studied extensively by Rovito and Kitrell (1973) with glucose oxidase immobilized on porous glass. Using packed beds containing beads of various particle sizes, the conversion rate was studied under conditions which minimized external film diffusion limitations. The smaller particles were found to be more active. It was calculated that in order to obtain a catalyst which was not diffusion limited particles of 30 microns diameter would have to be used compared with commercially available glass beads which have a diameter of 250 microns. These particles would be too small for use in large packed beds and an alternative is to use support materials with very large pore sizes. A material of this type, nickel impregnated silica aluminium, has been developed with a pore size ranging from 20,000 - 400,000 Å compared to the 2000 Å diameter of porous glass (Herring, Laurence and Kitrell, 1972).

Diffusion limitations can be detected by examination of Lineweaver-Burk plots. Very active derivatives of DEAE-cellulose-chymotrypsin gave non-linear reciprocal plots (Kay and Lilly, 1970), when the particles were ground to a smaller size, linear plots were obtained. Similar effects have been observed with glucose oxidase (Weibel and Bright, 1971) and with β -galactosidase (Regan, Dunnill and Lilly, 1974). Diffusion effects can also be observed through changes inthe activation energy. A decrease in activation energy from 11.1kcal/mole to 6.7kcal/mole was observed when aminoacylase was immobilized on DEAE-cellulose (Tosa et al., 1967). This has been ascribed to control of the reaction rate by pore diffusion.

Diffusional limitations will lead to high local concentrations of products and this can be used to advantage in immobilized multi-enzyme systems. A hexokinase - glucose 6-phosphate dehydrogenase system immobilized on Sepharose produces NADPH at a greater rate than the corresponding soluble system (Mosbach and Mattiasson, 1970). This can be attributed to a higher local concentration of the intermediate products. These results have been extended to a three enzyme system (Mattiasson and Mosbach, 1971). An immobilized multi enzyme system would seem to have advantages in the synthesis of complex molecules. All intermediates

would be retained in the microenvironment of the matrix and only the final product would diffuse into the medium.

Design of immobilized enzyme reactors

The two most common reactor configurations are the packed bed and the stirred reactor, which can be operated in either batch or continuous processes. The operating characteristics of packed beds and stirred reactors have been compared (Lilly and Sharp, 1968). In a system which is unaffected by product or substrate inhibition, a packed bed will be more efficient than the stirred reactor. Substrate inhibition will affect a packed bed more than a stirred reactor while product inhibition will have a more marked effect on the stirred tank.

The major cause of inefficiency in enzyme reactors is the loss of activity which can be due to two main causes.

1. Disintegration of the enzyme particle and loss from the reactor

During the operation of a stirred reactor containing AE-cellulose - β -galactosidase, it was found that the average size of the particle became smaller and particles were lost through the retaining filter. The smaller particles were less diffusion limited and their removal caused a disproportionate loss in activity (Regan, Dunnill and Lilly, 1974).

2. Denaturation of the enzyme in the reactor

An enzyme protected from thermal inactivation by substrate will be more effective in a packed bed reactor than in a stirred reactor. Conversely, an enzyme protected by product will operate more efficiently in a stirred reactor (O'Neill, 1972). For example, catalase is inactivated by hydrogen peroxide and if immobilized catalase was used in a packed bed, the enzyme would be rapidly inactivated. The inactivation can be minimized by using the enzyme in a stirred reactor.

Applications of immobilized enzymes

There are few examples of the use of immobilized enzymes on an industrial scale but there have been many pilot scale studies indicating possible uses.

Aminoacylase immobilized on DEAE-Sephadex is currently being used for the production of L-methionine from acetyl methionine

(Sato et al., 1971). Immobilized penicillin amidase can be used to prepare 6-aminopenicillanic acid from benzyl penicillin (Warburton, Dunnill and Lilly, 1973). The sequential conversion of steroids has been achieved using gel-entrapped 11 β-hydroxylase and Δ 1, 2 dehydrogenase (Mosbach and Larsson, 1970). Another major use of immobilized enzymes is in the analytical field and a wide variety of enzymes has now been bound to nylon tubes for use in automated analysis (Inman and Hornby, 1972). An immobilized glucose oxidase derivative has been used for 3500 assays while the same amount of soluble enzyme could only have been used for 1500 assays.

Almost all of the enzymes which have been immobilized are enzymes which carry out conversions of small substrate molecules or degrade macromolecules. One exception is polynucleotide phosphorylase which has been immobilized on cellulose and used in the large scale synthesis of polynucleotides (Hoffman et al., 1970). A major aim of the present project was to prepare a range of immobilized derivatives of polynucleotide phosphorylase and to study their properties.

Affinity Chromatography

The purification of an enzyme by conventional techniques which rely on small differences in physico-chemical properties such as solubility and charge is frequently a lengthy process. One advantage of the technique of affinity chromatography which has been developed in recent years is that the purification of an enzyme can be carried out in one step.

Affinity chromatography utilizes the specificity of an enzyme for its substrate. A ligand resembling the substrate is bound to an insoluble support and a column of this material is prepared. The crude enzyme solution is passed through the column, the enzyme is retained by the ligand while the remaining proteins are washed through. Elution of the enzyme can then be achieved in a number of ways, for example by a change in ionic strength, pH or temperature. The enzyme-ligand interaction can also be dissociated by washing the column with a solution of substrate or cofactor.

The purification of an enzyme by affinity chromatography was first reported in 1953 when tyrosinase was purified on a column of cellulose which had been treated with diazotized p-amino phenol (Lerman, 1953). The technique was also used for the isolation of antibodies (Campbell, Leuscher and Lerman, 1951). However, cellulose is not an ideal support material as non specific adsorption of macromolecules can occur. In addition, the material can break down to give fine particles, producing a column with a slow flow rate. The development of affinity chromatography as a routine technique has only occurred since suitable readily available materials became available.

The properties of a suitable matrix material can be described as follows: The matrix should not interact non-specifically with macromolecules, it should have good flow characteristics in column and it should be stable under a wide variety of conditions and the matrix should contain a large number of functional groups which can be modified or activated to allow covalent binding of a ligand. The material which is most commonly used is beaded agarose (Cuatrecasas, 1970;

Hjerten , 1962) which is readily activated by cyanogen bromide (Porath, 1968) and has a high capacity for ligand. Other materials which have been used in affinity chromatography are polyacrylamide (Inman and Dintzis, 1969) and porous glass (Weetall, 1973). The chemistry of the activation procedures have been discussed in the section on immobilized enzymes.

Selection of the ligand is perhaps the most critical step in the development of an affinity purification and the ligand must show a strong affinity for the enzyme. Early studies assumed that attachment of a ligand to the matrix would result in a decrease in affinity for the enzyme. However recent studies have indicated that this may not always be the case, as the affinity between lactate dehydrogenase and immobilized 5'-AMP is higher than the affinity between the free ligand and enzyme in solution (Lowe, Harvey and Dean, 1974). Similar results have been obtained with staphylococcal nuclease and an immobilized inhibitor (Dunn and Chaiken, 1974).

Other early studies indicated that the distance of the ligand from the matrix was important. This was demonstrated in the purification of tyrosine aminotransferase using immobilized pyridoxamine phosphate (Miller, Cuatrecasas and Thompson, 1972). When the cofactor was coupled directly to the matrix, there was no purification of the enzyme. When an eight carbon chain was introduced as a spacer between the cofactor and the matrix, there was a 650 fold purification of the enzyme. However, some workers have suggested that the enzyme could be interacting with the spacer chain and that the purification is not truly biospecific (O'Carra, Barry and Griffin, 1974). Glycogen phosphorylase has been purified using alkyl agarose derivatives (Er-El, Zaidenzaig and Shaltiel, 1972). The strength of binding was related to the length of the hydrocarbon chain and it was proposed that the hydrocarbon chains were interacting with hydrophobic pockets in the enzyme. Similar results have been obtained by Hofstee (1973) and the term hydrophobic chromatography has been introduced to describe these effects. However, hydrophobic interactions should be strengthed by an increase in ionic strength. In fact, enzymes were eluted from alkyl agaroses by increasing salt concentrations. It has been shown that when

an alkylamine is coupled to cyanogen bromide activated agarose, an ion exchanger is formed with a pK of 10 (Jost, Miron and Wilchek, 1974). The same authors proposed that the alkyl agarose derivatives were acting as detergents. The hydrophobic alkyl chains stick out away from the hydrophilic matrix and cause a partial unfolding of the polypeptide chain. The partial unfolding exposes charged groups in the protein which then interact with the charged matrix. The interaction between the enzyme and the spacer molecule can be minimized in a number of ways. Stable, high capacity derivatives can be prepared by coupling polyacrylic hydrazide to agarose (Wilchek and Miron, 1974) and have minimal ion exchange properties. Hydrophilic molecules such as 1,3, diaminopropanol can also be used as spacer molecules (O'Carra, Barry and Griffin, 1974). The structures of some of these derivatives are shown in Figure 8.

In view of these results, the advantages of the modular solid phase approach (Barry and O'Carra, 1973) should be reconsidered. In this approach, a spacer arm is attached to the matrix and the ligand is then coupled to the spacer molecule. The final product is not defined and if the spacer arms are not fully substituted, there is the possibility of hydrophobic interactions. The alternative approach is to assemble the ligand and spacer arm together and attach this to the matrix. The ligand assembly can be purified before coupling to the matrix and the resulting product is defined. This approach has been used successfully in the synthesis of AMP and NAD^+ analogues (Guilford, Larsson and Mosbach, 1972; Craven et al, 1974).

Normally, a specific ligand has to be developed for each enzyme that is to be purified. However, in some cases one ligand can be used to fractionate enzymes belonging to a particular group. For example, riboflavin cellulose derivatives have been used to purify three FMN dependent enzymes (Arsenis and McCormick, 1966). The concept of general ligand affinity chromatography has been developed for the NAD^+ dependent dehydrogenases (Mosbach et al., 1972; Lowe et al., 1973).

Although the main use of affinity chromatography has been in the purification of enzymes, the technique has also been used to study

FIGURE 8

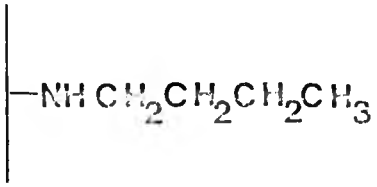
Spacer molecules used in affinity chromatography.

A. Hydrophobic spacer

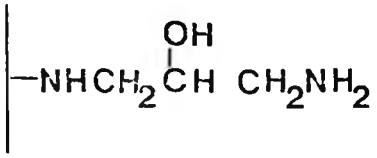
B. Hydropnilic spacer

C. Poly acrylic hydrazide

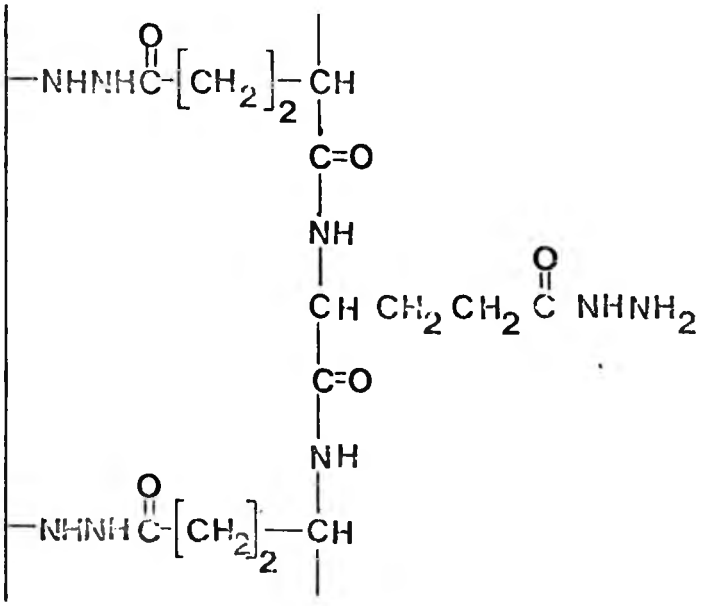
A



B



C



some properties of enzymes, for example, the binding of cofactor and substrate to lactate dehydrogenase using immobilized NAD^+ derivatives (O'Carra and Barry, 1972). Another use of the immobilized derivatives of nucleotide coenzymes has been their use in enzyme reactors as reusable cofactors. A soluble NAD^+-polyethyleneimine derivative has been prepared and retains its activity when used with immobilized alchchol dehydrogenase (Wykes, Dunnill and Lilly, 1972). Enzymatically active NAD^+ derivatives prepared with agarose (Lindberg, Larsson and Mosbach, 1974) and glass beads (Weibel, Wectall and Bright, 1971) have also been reported.

There are other techniques of enzyme purification which are closely related to affinity chromatography. For example, an enzyme bound to an ion-exchange matrix can sometimes be eluted with substrate. Fructose 1,6-diphosphatase has been eluted from CM-Sephadex using either block elution with substrate of a substrate gradient (Sarngadharan, Watanabe and Pogell, 1970). Similar results have been obtained with glucose 6-phosphate dehydrogenase and t-RNA amino acyl synthetases. An advantage of the technique is that the ion exchange matrix is readily available and there is no need to prepare an affinity ligand.

Experimental

Materials

Nucleoside diphosphates and polynucleotides were obtained from P-L Biochemicals. Cl^5 CDP and ho^5 CDP were prepared by Dr. M.A. Eaton following published procedures (Eaton and Hutchinson, 1972, 1973). E. coli t-RNA was purchased from BDH Chemicals Ltd and yeast t-RNA (phenylalanine specific) was purchased from Boehringer. $[^{14}C]$ - labelled nucleotides, with the exception of $[^{14}C]$ - IDP, were the products of the Radiochemical Centre, Amersham. $[^{32}P]$ - CTP was obtained from the same source.

Lactate dehydrogenase (E.C.1.1.1.27) type III, pyruvate kinase (E.C.2.7.1.40) type II, deoxyribonuclease I (E.C.3.1.4.5) ribonuclease free, lysozyme (E.C.3.2.1.17) and trypsin (E.C.3.4.4.4) were purchased from Sigma.

Sepharose and Sephadex derivatives were the products of Pharmacia. DEAE-cellulose (DE23) and cellulose (CF12) were purchased from Whatman Biochemicals Ltd. Porous glass beads and Celite-560 were obtained from BDH Chemicals.

Polyethyleneimine, 1,6-diaminohexane and trinitrobenzene sulphonic acid were obtained from BDH Chemicals and were used without further purification. m-Aminophenylboronic acid hemisulphate was purchased from Ralph Emmanuel and was purified by recrystallisation from dioxan. All other chemicals were of Analar grade.

Micrococcus luteus (ATCC 4698) was obtained as a spray dried powder from Miles-Seravac. Escherichia coli B and Bacillus stearothermophilus were obtained as frozen cell pastes from the Microbiological Research Establishment, Porton.

Methods

Activation of Sepharose-4B with cyanogen bromide (Axen and Ernback, 1971)

Sepharose-4B (25 ml) was suspended in an equal volume of water, cyanogen bromide (5g) was added and the suspension stirred vigourously, the pH of the suspension being maintained at 11.0 by the dropwise addition of 8 M sodium hydroxide. After ten minutes, the suspension was filtered and washed with cold 0.20 M sodium bicarbonate (500 ml). The activated material was then used immediately. Alternatively, the method of March, Parikh and Cuatrecasas (1974) was used. Sepharose-4B (25 ml) was suspended in an equal volume of water. 2 M sodium carbonate (50 ml) was added to the suspension which was then stirred rapidly. A solution of cyanogen bromide (5g) in dry acetonitrile (2.5 ml) was added and after two minutes, the suspension was filtered and washed as before. The concentration of cyanogen bromide can be varied but for routine experiments a concentration of 2g/ml acetonitrile was used. The advantages of this method are the shortened activation time and the simplicity of the procedure.

Preparation of ligands for affinity chromatography

Preparation of aminohexyl Sepharose

1,6-Diaminohexane (5g) was dissolved in water (25 ml) and the pH of the solution was adjusted to 10.0. An equal volume of activated Sepharose-4B was added and the suspension was stirred for twenty hours at 4^{o}. The gel was then washed on a filter with water (500 ml), 1 M sodium chloride (500 ml) and water (500 ml). The concentration of bound ligand was determined by reaction with trinitrobenzenesulphonic acid (TNBS) (Newirth et al., 1973).

Preparation of succinylated Sepharose

Aminohexyl Sepharose (5.0 ml) was suspended in an equal volume of water, succinic anhydride (5.0g) was added and the pH adjusted to 6.0. The suspension was stirred for eighteen hours at 4^{o} and then washed thoroughly. The gel gave a negative response to the TNBS indicating complete substitution of the amino groups.

Reaction of ADP and succinylated Sepharose

Adenosine diphosphate (Na^+ salt, 100 mg) was dissolved in water (10 ml) and added to succinyl Sepharose (4.0 ml). The pH was adjusted to 5.0 and the suspension was stirred while an aq. solution of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulphonate (3.0 ml, 70 mg/ml) was added dropwise. The pH was readjusted to 5.0 and the suspension was stirred at room temperature for twenty-four hours. The gel was filtered and washed as before. The concentration of bound ligand was estimated from the UV absorbance of the washings. Similar procedures were used for the preparation of immobilized CDP and dADP derivatives.

Preparation of succinyl ADP

ADP (Na^+ salt, 200 mg) was dissolved in dry DMSO (40 ml), succinic anhydride (8g) was added and the solution was stirred at room temperature for forty-eight hours when the nucleotide was precipitated by the addition of acetone. The precipitate was collected by centrifugation, washed with acetone and dissolved in water (2.5 ml). The pH of the solution was adjusted to 8.0 and the solution was applied to a column of Dowex 1, formate form (1.0 x 20 cm). The modified nucleotide was eluted with a linear gradient of formic acid (0-0.25 M). Fractions containing succinyl ADP were pooled and lyophilized.

Coupling of succinyl ADP to aminohexyl Sepharose

Succinyl ADP (150 mg) in water (10 ml) was added to a suspension of aminohexyl Sepharose (5.0 ml). The pH was adjusted to 5.0 and an aqueous solution of water soluble carbodiimide (3 ml, 70 mg/ml) was added dropwise to the stirred suspension. After twenty-four hours at room temperature, the gel was filtered and washed as described before.

Immobilization of ADP on a diazonium derivative of agarose

Aminohexyl Sepharose (5 ml) was suspended in 0.20 M sodium borate pH 9.3 (20 ml) containing 40% DMF (V/V) and 0.10 M p-nitrobenzoyl azide and stirred at room temperature for sixty minutes. The gel was washed with DMF (250 ml) and gave a negative test with TNBS. The gel was then suspended in 0.10 M sodium dithionite in 0.50 M sodium bicarbonate,

pH 8.5 (20 ml). After forty-five minutes at 40°, the gel was washed on a filter and suspended in cold 0.50 M hydrochloric acid (5 ml). Sodium nitrite (100 mg) was added and the suspension stirred for ten minutes at 4°. The gel was washed on a filter with cold water (500 ml) and was added to 0.20 M sodium borate, pH 8.5 (10 ml) containing ADP (200 mg). The pH was readjusted to 8.5 and the suspension stirred for sixteen hours at 4° after which the gel was filtered and washed. The azo-bond between ligand and matrix can be reduced by sodium dithionite giving an accurate determination of the concentration of ligand on the matrix.

Preparation of immobilized polynucleotides

Activated Sepharose-4B (5 ml) was suspended in 0.05 M potassium phosphate, pH 8.0 (5 ml) containing polynucleotide (100 A260 units). The suspension was stirred at 4° for eighteen hours and the gel was then washed. The concentration of bound ligand was estimated from the UV absorbance of the washings.

Preparation of p-aminophenyl oligo (dT)

Oligo deoxythymidylic acid (oligo (dT)) was prepared by chemical polymerization of thymidine monophosphate (Tener et al., 1958; Khorana and Vizsolyi, 1961. Oligo (dT) (pyridinium salt, 100 mg) was dissolved in dry pyridine (5 ml), recrystallized p-nitrophenol (0.60 g) was added and the residue dried by repeated evaporation of pyridine (3 X). The residue was dissolved in dry pyridine (10 ml) and dicyclohexyl carbodiimide (0.80 g) added and the mixture was then stirred for seventy-two hours at room temperature in the dark. The pyridine was evaporated under vacuum and the residue dissolved in water. The solution was extracted with ethyl acetate (4 x 10 ml) and the aqueous layer evaporated to dryness.

The p-nitrophenyl ester of oligo(dT) was reduced by catalytic hydrogenation using palladium on charcoal (10% W/W, 50 mg) in 50% aqueous methanol (50 ml). The reduction was carried out at room temperature for ninety minutes at 35 psi after which the catalyst was removed by filtration and the solution evaporated to dryness. The oligonucleotide was purified on Whatman 3 MM paper with 1 M ammonium acetate-ethanol (1:1) as solvent. The p-aminophenyl oligo (dT) remained

at the origin and was eluted from the paper with 0.20 M sodium bicarbonate, pH 8.6.

Preparation of immobilized p-aminophenyl oligo (dT)

Activated Sepharose-4B (350 ml) was suspended in an equal volume of 0.20 M sodium bicarbonate, pH 8.6 containing the oligonucleotide ligand (7000 A260). After eighteen hours at 4°, the gel was washed with cold water (2.0 litres), 5 M sodium chloride (2.0 litres) and water (2.0 litres). The gel was stored as a suspension in water at 4° in the presence of sodium azide.

Preparation of polynucleotide phosphorylase

In later studies, the enzyme was isolated by affinity chromatography but in early work, the enzymes were isolated by modifications of published methods.

Isolation of polynucleotide phosphorylase from M. luteus (Thanassie and Singer, 1966)

M. luteus cells (25g) were suspended in 0.5% sodium chloride (250 ml) at 37°. The pH was adjusted to 8.0 with 1.0 M tris. Lysozyme (100 mg in 4 ml water) was added and the suspension stirred for ten minutes. Cold saturated ammonium sulphate solution (125 ml) was added and the suspension was centrifuged for fifteen minutes at 20,000 x g to remove cell debris. The supernatant was bought to 65% saturation with ammonium sulphate. The precipitate was collected by centrifugation (fifteen minutes, 20,000 x g) and dissolved in 0.10 M tris$^{\cdot HCl}$ pH 8.1 and dialysed against the same buffer for four hours at 4°.

The solution was diluted with an equal volume of 0.10 M tris$^{\cdot HCl}$ pH 8.1 and bought to 40% saturation with ammonium sulphate. After thirty minutes at 4°, the solution was centrifuged (twenty minutes, 20,000 x g) and the precipitate discarded. The solution was then bought to 55% saturation with ammonium sulphate and the precipitate was collected by centrifugation. The precipitate was dissolved in the minimum amount of 0.10 M tris$^{\cdot HCl}$ pH 8.1 and dialysed against 0.10 M tris$^{\cdot HCl}$ 1 mM EDTA, pH 8.1 for four hours at 4°. Streptomycin sulphate solution (10%) was added to the solution to a final concentration of 0.45%. The solution was stirred for thirty minutes and then nucleic acids were removed by centrifugation

(ten minutes, 20,000 x g).

The supernatant was dialysed against 0.01 M tris^{-HCl} pH 8.0 for two and a half hours and was then adjusted to pH 6.3 with 1.0 M acetic acid. Ammonium sulphate was added to 42% saturation, the solution was centrifuged and the precipitate discarded. The supernatant was then bought to 52% saturation with ammonium sulphate. The precipitate was collected by centrifugation and dissolved in the minimum amount of 0.50 M tris^{-HCl} pH 8.0 and dialysed against 0.01 M tris^{-HCl} 1 mM EDTA, pH 8.0 for twelve hours at 4°. This fraction could be stored at -20° for up to twelve months and was sufficiently pure for the preparation of polynucleotides. The enzyme can be further purified by precipitation of nuclease with zinc chloride and gel filtration on G-75 Sephadex. In a typical experiment, 0.10 volumes of 0.10 M zinc chloride solution was added to a solution of the enzyme. The solution was stirred for twenty minutes at 4° and the precipitate was removed by centrifugation. The supernatant (5.0 ml) was applied to a column of Sephadex G-75 (2.50 x 60 cm) equilibrated with 0.01 M tris^{-HCl} 0.001 M EDTA, pH 8.2 containing 0.25 M sodium chloride. The column was eluted with the same buffer, active fractions eluted at the void volume and were pooled and stored at -20°.

Polynucleotide phosphorylase from E. coli (Kimhi and Littauer, 1968; Portier et al., 1973).

E. coli B cells (54g wet weight) were suspended in 0.01 M tris-HCl, 0.01 M magnesium chloride, pH 7.4 (40 ml). Glass beads (0.45 - 0.50 mm diameter, 54g) and silicone antifoam liquid (1 ml) were added and portions of the suspension (10 ml) were homogenized in a Braun shaker for forty-five seconds at 4000 rpm. Buffer (170 ml) was added and the suspension was stirred for twenty minutes at 4° and then centrifuged (16,000 x g, twenty minutes). The supernatant was stored at 4° while the cell debris and beads were re-extracted with buffer (100 ml).

The two supernatants were combined and heated at 55° for five minutes. The solution was rapidly cooled and the precipitate was removed by centrifugation (twenty minutes, 20,000 x g). Streptomycin sulphate solution (pH 7.4, 10%) was added to the supernatant to a final

concentration of 0.50%. After ten minutes, nucleic acids were removed by centrifugation (ten minutes, 10,000 x g).

Ammonium sulphate was added to the supernatant to 39% saturation and the precipitate was collected by centrifugation (ten minutes, 16,000 x g) and discarded. The supernatant was bought to 55% saturation and the precipitate collected by centrifugation, dissolved in 0.01 M tris-HCl, pH 7.4 and dialysed against the same buffer for twelve hours.

The solution was adjusted to pH 6.5 with 1.0 M acetic acid and precooled acetone (-20°) was added to 39% saturation. During the addition of the acetone, the solution was stirred to maintain even mixing and the temperature was maintained at -5°. After addition of the acetone, the solution was stirred for a further ten minutes and then centrifuged, (five minutes, 20,000 x g). Acetone was added to a final concentration of 49% and the precipitate was collected by centrifugation dissolved in 0.02 M tris-HCl, pH 7.5 and dialysed against the same buffer for six hours. This fraction could be used for the preparation of polynucleotides but could be further purified by gel filtration on Sephadex G-200. The enzyme could be stored at -20° for up to twelve months.

Preparation of enzyme extracts from E. coli and B. stearothermophilus for affinity chromatography

Cells (100 g wet weight) were suspended in 0.10 M tris-HCl, pH 8.0 (500 ml) containing 0.5% sodium chloride. Lysozyme (50 mg in 4 ml buffer) was added and the suspension was stirred at 37° for sixty minutes (Hachimori, Muramatsu and Nosoh, 1970). Deoxyribonuclease (1 mg) was added and the solution stirred for a further ten minutes. The solution was centrifuged (10,000 x g, twenty minutes) to remove cell debris. Polyethyleneimine solution (10%, pH 7.5) was added to the supernatant to a final concentration of 0.30% (Atkinson and Jack, 1973). This step precipitates enzyme activity with the nucleic acids. The precipitate was collected by centrifugation and resuspended in 0.01 M tris-HCl, 0.50 M potassium chloride, 1 mM 2-mercaptoethanol, pH 8.0. The solution was bought to 70% saturation with ammonium sulphate. After onehour at 4°, the precipitate was collected by centrifugation, dissolved in 0.01 M tris-HCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 8.0 and applied to a column of

Sepharose-4B (2.5 x 40 cm) which had been equilibrated with the same buffer. Active fractions were pooled and stored at -20°.

Binding of polynucleotide phosphorylase to CM-Sephadex and elution by substrate

CM-Sephadex C-50 was precycled with 0.50 M sodium hydroxide and 0.50 M hydrochloric acid, packed in a column (1.0 x 9.0 cm) and equilibrated with 5 mM MOPS, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 6.5 at 4°. A sample of enzyme was dialysed against the same buffer and applied to the column. The column was then washed with buffer until no protein was eluted. The column was then perfused with a buffer solution containing 1 mM ADP and fractions were collected and assayed for activity.

Immobilization of polynucleotide phosphorylase on insoluble support materials

Binding of polynucleotide phosphorylase to cyanogen bromide activated polysaccharides

Before use, cellulose (Whatman CF12) was mercerized in 5M sodium hydroxide for twelve hours at 4°. The cellulose was then washed with water until the washings were neutral. Sepharose and Sephadex were used without pre-treatment. The materials were activated as described previously.

The activated material (100 mg) was added to a solution of the enzyme (5 ml, 4 mg protein/ml) which had been dialysed against 0.01 M collidine, pH 8.0. The suspension was stirred at 4° for eighteen hours and then packed in a column and washed with 1.0 M sodium chloride (250 ml), 0.10 M sodium bicarbonate (250 ml), water (250 ml) and 0.10 M tris^{-HCl} pH 8.0 (250 ml). The conjugate was stored at 4° in 0.10 M tris^{-HCl} pH 8.0.

Binding of polynucleotide phosphorylase to syn-triazinyl activated polymers

Preparation of 2-amino-4, 6-dichloro-s-triazine (Thurston et al, 1951)

Cyanuric chloride (50g) was dissolved in a mixture of dioxan (550 ml) and toluene (110 ml) and stirred vigorously in an ice bath. Dry ammonia was blown through the solution for four hours when the solution was purged with nitrogen until free of ammonia. The solution was then filtered and the filtrate evaporated to dryness. The residue was dissolved in acetone

(500 ml) and an equal volume of water was added to the solution. The volume was reduced to a half by evaporation and the crystals which formed were collected by filtration. The product (40g) was stored in a desiccator at room temperature.

Preparation of DEAE-cellulose-amino chloro-s-triazine (Kay and Lilly, 1970)

2-Amino-4, 6-dichloro-s-triazine (4g) was dissolved in aqueous acetone (50% V/V) (200 ml) at 50°. DE23 cellulose (20g) was added and the suspension stirred vigorously for sixty seconds. A solution of 15% sodium carbonate (16 ml) containing 0.6 volume of 1 M hydrochloric acid was added and the stirring continued for five minutes when the solution was made acid by the addition of concentrated hydrochloric acid. The activated cellulose was collected by filtration and washed with acetone (200 ml) and water (200 ml). The activated polymer was stored in 0.10 M phosphate buffer, pH 7.5 at 4°.

Coupling of polynucleotide phosphorylase to activated DEAE-cellulose

Activated polymer (2g) was washed on a filter with 0.10 M sodium borate, pH 8.5 and was then added to a solution of enzyme (10ml, 4 mg/ml) which had been dialysed against 0.10 M sodium borate pH 8.5. The suspension was then stirred at 4° for eighteen hours and then washed as described for the cyanogen bromide activated conjugates.

Binding of polynucleotide phosphorylase to inorganic supports

Porous glass beads (CPG-10, 200-400 mesh, 1400 A° pore diameter) and Celite-560 were washed with concentrated nitric acid and then with distilled water. The beads were derivatized by the method of Robinson, Dunnill and Lilly (1971). Beads were suspended in a 2% solution of γ-aminopropyltriethoxysilane in acetone, excess solvent was decanted and the beads maintained at 45° for eighteen hours. The beads were then washed with acetone and stored in a desiccator until required for use. In an alternative procedure, the beads were suspended in a 10% solution of γ-aminopropyltriethoxysilane in toluene and refluxed for eighteen hours. The beads were then washed with acetone, dried and then refluxed for a further eighteen hours.

The alkylamine beads (2g) were suspended in 5% aqueous glutaraldehyde (20 ml) with gentle stirring at 4° for one hour. The beads were then washed with cold water (100 ml) and added to a solution of enzyme (10 ml, 4 mg/ml) in 0.10 M borate, pH 8.5. The suspension was stirred at 4° for two and a half hours when the derivative was packed in a column and washed as described for the other immobilized enzyme derivatives.

Modification of immobilized polynucleotide phosphorylase by trypsin

Immobilized enzyme was suspended in water (25 ml) in a jacketed vessel which was maintained at 25°. The pH of the suspension was adjusted to 8.0 and solution of trypsin (0.50 ml, 0.40 mg/ml) was added. The pH of the stirred suspension was maintained at 8.0 by the addition of 0.05 M sodium hydroxide. When there was no further change in pH, a solution of 250μM phenylmethylsulphonyl fluoride (1.0 ml) was added to inhibit the protease (Fahrney and Gold, 1962) and the enzyme conjugate was washed with 1.0 M sodium chloride, 0.10 M sodium bicarbonate and water. The conjugate was then stored in polymerization buffer at 4°.

Determination of the protein content of immobilized enzyme derivatives

A crude measurement of the protein content can be made by measuring the amount of unbound protein in the washings. A more accurate value can be obtained by acid hydrolysis followed by determination of the amino acids with ninhydrin (Crook, Brocklehurst and Wharton, 1970).

A sample of immobilized enzyme was placed in a hydrolysis tube and 6 M hydrochloric acid was added. The tube was sealed and heated at 110° for forty-eight hours. A standard was prepared by hydrolysing a known amount of enzyme under similar conditions. The samples were cooled and filtered and the filtrates evaporated to dryness and made up to 10 ml with water. Aliquots (1 ml) were taken and 0.20 M citrate, pH 5.0 (0.50 ml), ninhydrin (5% W/V in 2-methoxyethanol) (0.20 ml) and 0.01 M potassium cyanide in 2% 2-methoxyethanol (1 ml) were added. The samples were heated at 100° for fifteen minutes and then cooled rapidly. If necessary, the samples were diluted with 60% ethanol before measuring the absorbance at 570 nm. A standard curve was prepared at the same time using the known enzyme sample.

Determination of enzymic activity

Soluble enzyme

Polymerization was measured by following the incorporation of $[^{14}C]$-ADP into polymeric material (Eaton and Hutchinson, 1972). The activity of E. coli and M. luteus polynucleotide phosphorylase was measured at 37° in 0.10 M tris^{-HCl}, 10 mM magnesium chloride, 1 mM EDTA, 2 mM 2-mercaptoethanol pH 9.2. The enzyme from B. stearothermophilus was assayed at 70° in a 0.10 M glycine-sodium hydroxide buffer, pH 9.2, containing 10 mM magnesium chloride, 1 mM EDTA and 2 mM 2-mercaptoethanol. In both cases, the final substrate concentration was 10 mM. One unit of activity is defined as the amount of enzyme which will incorporate 1 μ mole of ADP into polymeric material in one hour.

Phosphorolysis was measured by the release of $[^{32}P]$ - labelled nucleoside diphosphate (Chou and Singer, 1970a). The enzymes from M. luteus and E. coli were assayed at 37° in 0.10 M tris^{-HCl}, pH 8.2 containing 10 mM potassium phosphate, 0.5 mM magnesium chloride, 2 mM mercaptoethanol and 1 mM poly(A).

Before use, all buffers were degassed, purged with nitrogen and degassed again. Protein was measured by the method of Lowry et al (1951).

Immobilized enzyme

Immobilized enzyme derivatives were assayed in stirred suspension or in a packed bed reactor. Stirred suspension assays were carried out in a jacketed glass reaction vessel. The stirring rate was sufficient to maintain the enzyme particles in suspension but low enough to avoid vortexing. Packed bed reactors were assayed using a jacketed column and a peristaltic pump. Substrate solutions were maintained at constant temperature and at least two column volumes were passed through the reactor before assay.

Polymerization was measured by release of phosphate (Ames and Dubin, 1960). Aliquots were removed from the reaction mix and filtered, made up to 1 ml with 1 M hydrochloric acid and added to 1 ml of phosphate reagent. This reagent was prepared daily and contained one

volume of 10% ascorbic acid, two volumes of water, one volume of 2.5% ammonium molybdate and one volume of 6 N sulphuric acid. Samples were incubated at 45° for twenty minutes and the absorbance was then measured at 820 nm.

Phosphorolysis was assayed by measuring the release of ADP from poly(A) using a linked enzyme system (Ochoa and Mii, 1961) in which the reaction was followed by observing the oxidation of NADH. A typical assay contained in 1 ml, potassium phosphate 10 μmole, magnesium chloride 5 μmole, EDTA 1 μmole, phosphoenol pyruvate 1.6 μmole, NADH 0.13 μmole, poly(A) 0.18 μmole, lactate dehydrogenase 0.30 units and pyruvate kinase 0.30 units. Immobilized enzyme in 0.10 M tris^{-HCl} pH 8.0 was placed in the reaction vessel. The reagents, with the exception of poly(A) were added. After equilibration, the poly(A) was added and aliquots removed for measurements.

Initial velocities were taken from linear portions of the progress curves. Reciprocals of the initial velocity were plotted against the reciprocal of substrate concentration to check that the data fitted the Michaelis Menten equation.

$$v = \frac{V_{max.}\ S}{K_m + S} \qquad (1)$$

Kinetic constants were determined from the intial velocity and substrate concentration data pairs using a computer programme described by Wilkinson (1961). Values of Km and Vmax were obtained by weighted regression of the recip rocal form of equation 1.

Detection of ribonuclease activity in purified enzyme (Kimhi and Littauer, 1968)

A sample of enzyme was incubated in the presence of $\left[^{14}C\right]$ - poly(C) in polymerization buffer for eight hours at 37°. An aliquot was applied to a cellulose TLC plate which was developed in 1 M ammonium acetate-ethanol (1:1). The plate was cut into strips and radioactive products were detected by scintillation counting.

Detection of proteolytic enzymes

Aliquots of the enzyme were assayed for esterase activity

against BAEE and ATEE (Schwert and Takenaka, 1955). Peptidase activity was measured using carbobenzoxyglycyl-L-phenylalanine as substrate (Petra and Neurath, 1969). Amidase activity was measured against BAPNA (Erlanger, Kokowsky and Coke, 1961). Assays were carried out both in the presence and absence of 2 mM 2-mercaptoethanol.

Preparation of m-aminobenzeneboronic acid - Sepharose

Sepharose-4B (50 ml) was activated by cyanogen bromide according to the method of March et al (1974) and was added to a solution of recrystallised m-aminophenylboronic acid in 0.10 M phosphate buffer, pH 6.5 (50 ml, 20 mg/ml). The suspension was stirred at 4° for eighteen hours and was then washed on a filter with 1.0 M sodium chloride (500 ml), water (500 ml), 1.0 M sodium chloride (500 ml) and water (500 ml). The washings were pooled and their absorbance at 293 nm was measured to determine the extent of binding.

Isolation of oligonucleotides with intact 3'-hydroxyl groups on a column of dihydroxylboryl Sepharose

The procedure of Rosenberg (1974) was followed. The Sepharose derivative was packed in a column (1 x 10 cm) and equilibrated with Buffer A (20% DMSO, 0.05 M morpholine, 1.0 M sodium chloride, 0.10 M magnesium chloride, pH 8.5) at room temperature. The sample of oligonucleotide was dialysed against Buffer A and applied to the column. Unbound material was eluted from the column with Buffer A and the oligonucleotides bearing 3'-hydroxyl groups were eluted with Buffer B (20% DMSO, 0.05 M MOPS, 1.0 M sodium chloride, pH 5.5). The oligonucleotide was then dialysed against 1 mM EDTA and finally against water. The material was lyophilized and stored at -20°.

Preparation of $\left[^{32}P\right]$ - CDP

$\left[^{32}P\right]$ - CDP was prepared from $\left[^{32}P\right]$ - CTP by a modification of the method of Tetas and Lowenstein (1963). $\left[^{32}P\right]$ - CTP (250 μcuries, 9.14 curies m mole^{-1}) was dissolved in 20 mM cupric sulphate in 0.10 M sodium acetate, pH 5.0 (50 μl). The solution was incubated at 80° for fifteen minutes when the reaction was stopped by the addition of 0.10 M EDTA (50 μl). The solution was applied to Whatman 52 paper and the product

was purified by electrophoresis in a pyridinium acetate pH 3.5 system. The product was identified by autoradiography and eluted with 30% triethylammonium carbonate, pH 10.3.

Preparation of $\left[^{14}C\right]$ - IDP

Sodium nitrite (5 mg) was added to a solution of ADP (1 mg/50 l) containing 10μCi of $\left[^{14}C\right]$-ADP in 10% acetic acid at 0°. The container was sealed and kept at 0° for twenty four hours before removal of solvent under vacuum. The product was dissolved in water (25μl) and applied to a cellulose TLC plate which was developed in isobutyric acid/ammonia/water (66/1/33 V/V). The product was obtained in quantitative yield and moved with an Rf = 0.13 which was identical to the Rf of authentic IDP. The product was eluted from the cellulose with 0.50 M triethylammonium bicarbonate.

Isolation of oligo(I) primers

Poly(i) (100 mg) was dissolved in 0.02 MOPS, 20 mM magnesium chloride, pH 7.2 (10 ml) containing pig liver nuclease (500 units). After two hours at 37°, the solution was deproteinized with isoamyl alcohol-chloroform (2:5 V/V) (Scheit and Gaertner, 1969) and applied to a column of Sephadex G-200 (1.6 x 6 cm) which had been equilibrated with 0.10 M triethylammonium bicarbonate at 4°. Oligonucleotides were eluted with the same buffer and fractions corresponding to a particular size class were pooled and lyophilized. Oligonucleotides were characterized by phosphate analysis and by gel electrophoresis.

Incorporation of CDP into oligo(I) primers

Each reaction contained 2 A_{250} units of oligonucleotides, 0.6 μmole $\left[^{3}H\right]$-CDP, 40 μg *B. stearothermophilus* polynucleotide phosphorylase in 0.10 M glycine, pH 9.2, (100μl) containing 10 mM magnesium chloride, 1 mM EDTA and 1 mM 2-mercaptoethanol. The reaction was carried out at 70° and 5 μl aliquots were removed for analysis at regular intervals.

Preparation of $\left[^{32}P\right]$ labelled t-RNA-oligo(C)

CDP (0.5μmole containing 10 μCi $\left[^{32}P\right]$-CDP) was incubated with phenylalanine specific t-RNA from brewers yeast (5×10^{-9} mole) and *B. stearothermophilus* enzyme (10 units) in 0.10 M glycine pH 9.0 (200μl) containing 5 mM manganous chloride and 2 mM 2-mercaptoethanol for

thirty minutes at 65°. The reaction mixture was neutralized by the addition of 1.0 M sodium acetate, pH 5.5 (200μl) and deproteinized with isoamyl alcohol-chloroform. The aqueous solution was applied to a column of Sephadex G-50 (0.9 x 50 cm) which had been equilibrated with 0.10 M triethylammonium bicarbonate, pH 7.5 at 4°. The modified t-RNA eluted at the void volume and the solution was lyophilized.

Characterization of $[^{32}P]$ labelled t-RNA

The material was dissolved in 0.02 M tris, pH 7.4 (10μl) containing 1 mM EDTA and pancreatic ribonuclease (Enzyme : Substrate = 1 : 20) and was incubated for sixty minutes at 37°. The sample was lyophilized and dissolved in water (2 μl) and applied to a strip of cellulose acetate and electrophoresed in pyridinium acetate pH 3.5. Products were then separated in the second dimension by electrophoresis on DEAE paper in 7% formic acid, pH 1.7 and were identified by autoradiography.

Products of the ribonuclease digestion were further characterized by alkaline hydrolysis. Products were eluted from DEAE paper with 30% triethylammonium carbonate and lyophilized. The samples were then dissolved in 0.4 M potassium hydroxide (10μl) and incubated in sealed capillary tubes for eighteen hours at 37°. The samples were then applied to Whatman 52 paper and products separated by electrophoresis in a pyridinium acetate, pH 3.5 system.

Results and Discussion

Purification of polynucleotide phosphorylase

The preparation of the enzyme from M. luteus has been adapted from the procedure of Singer (1966). Streptomycin sulphate was used in place of protamine sulphate for the precipitation of nucleic acids, as it was found that some batches ofprotamine sulphate contained protease and nuclease impurities. Streptomycin sulphate also has the advantage of being removed on dialysis. The preparation of the E. coli enzyme is based on the methods of Kimhi and Littauer (1969). A heat step at 55^{o} was included at an early stage in place of the autolysis step of the original paper. The heat step removes a significant amount of protein and minimizes proteolytic activity. The purifications are outlined in Tables 2 and 3. In both cases, fraction 4 was suitable for the preparation of polynucleotides.

The purification of polynucleotide phosphorylase by conventional methods is a lengthy procedure during which the enzyme can be modified by proteolytic enzymes. An attempt was made to devise an affinity chromatography purification in order to simplify the purification and to minimize proteolytic degradation. Immobilized derivatives of ADP and dADP were prepared for use as affinity ligands since ADP is a substrate of the enzyme and dADP is a competitive inhibitor (Chou and Singer, 1971). Several approaches to the synthesis of these derivatives were tried and are outlined in Figures 9 - 11.

As shown in Figure 9, it was hoped to prepare a Schiff's base between p-nitrobenzaldehyde and the N-6 amino group of the adenine ring. The nitro group and the Schiff's base could then be reduced to an amine group which could be coupled directly to cyanogen bromide activated Sepharose. The benzene ring would provide a useful spacer and the phosphate groups of the nucleotide would be free for binding to the enzyme. Unfortunately this approach was unsuccessful as the N-6 amino group was not sufficiently reactive.

A second approach was to succinylate the N-6 amino group of the nucleotide and to react the succinyl-ADP with 1, 6-diamino hexane. The ligand-spacer complex could then be reacted with activated Sepharose.

An alternative approach was to prepare a succinylated derivative of aminohexyl Sepharose and then to react this with ADP using a water soluble carbodiimide (Figure 10). This method is less satisfactory as there is incomplete substitution of the amino groups leading to possible non-specific hydrophobic interactions. Derivatives of ADP, dADP and CDP were prepared by these methods but they did not bind or retard polynucleotide phosphorylase under the conditions used. The method has been used to prepare NAD^+ - polyethyleneimine derivatives (Wykes, Dunnill and Lilly, 1972). The linkage between NAD^+ and polyethyleneimine was found to be unstable at alkaline pH and since the immobilized ADP derivatives were used in the range pH 7 - 9 there could have been a loss of nucleotide from the columns.

A third approach was to react the nucleotides with a diazonium-Sepharose derivative. It has been reported that reaction of the purine nucleotides with diazonium salts leads to substitution at the C-8 position (Hoffman and Muller, 1966). Immobilized derivatives of ADP were prepared by this method but were not effective in the purification of polynucleotide phosphorylase. A possible reason for the ineffectiveness of the derivatives is the fact that a bulky substituent at the C-8 position prevents the nucleotide from assuming the anti conformation. The nucleotide must be in the anti conformation for polymerization to take place as nucleotides in the syn conformation can bind to the enzyme but will only polymerize with difficulty (Howard, Frazier and Miles, 1975; Kapuler and Reich, 1971).

Immobilized nucleotides have been prepared in which the nucleotide is bound to the matrix through the 5' phosphate groups (Barker et al., 1972). However, it has been shown that polynucleotide phosphorylase has a binding site for the phosphate groups of a nucleotide (Chou and Singer, 1970b) and derivatives prepared by the method of Barker et al. (1972) would be unsuitable as affinity ligands for polynucleotide phosphorylase.

As ligands based on mononucleotides were ineffective in the purification of the enzyme, it was decided to prepare immobilized derivatives of oligonucleotides and polynucleotides. Agar gels containing DNA retard but do not bind polynucleotide phosphorylase (Weatherford et al., 1972). However, the DNA was not covalently linked to the matrix and the material

TABLE 2

Purification of Polynucleotide Phosphorylase from M. luteus

Step	Protein (mg/ml)	Specific Activity (units/mg)	Recovery %
1. Crude Extract	2.0	0.60	100
2. 40-55% Ammonium Sulphate	20	2.50	92
3. Streptomycin Sulphate	5.0	7.00	90
4. 40-55% Ammonium Sulphate, acid pH	15.0	9.0	59
5. Zinc Chloride G-75 Sephadex	0.20	200	45

TABLE 3

Purification of Polynucleotide Phosphorylase from E. Coli

Step	Protein(mg/ml)	Specific Activity (units/mg)	Recovery
1. Crude Extract	40	0.55	100
2. Heat Step	20	0.90	80
3. Ammonium Sulphate	15	2.70	75
4. Acetone	14	9.00	60
5. G-200 Sephadex	0.30	400	25

FIGURE 9

Reaction of adenosine derivatives with p-nitrobenzaldehyde.

Formation of a Schiff's base followed by catalytic hydrogenation.

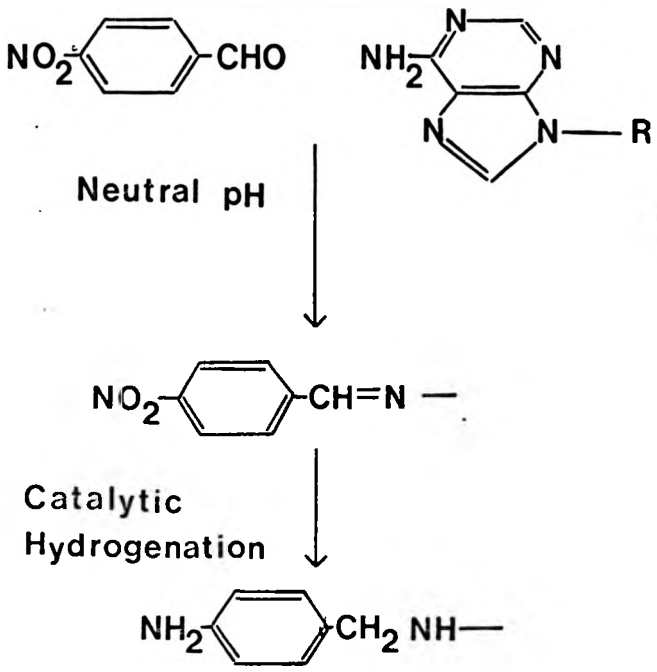

NO2
CHO
NH2
N
N
N
N
R
Neutral pH
NO2
CH=N —
Catalytic
Hydrogenation
NH2
CH2 NH—

FIGURE 10

Reaction of aminohexyl-Sepharose with succinic anhydride and subsequent reaction with ADP and dicyclohexyl carbodiimide.

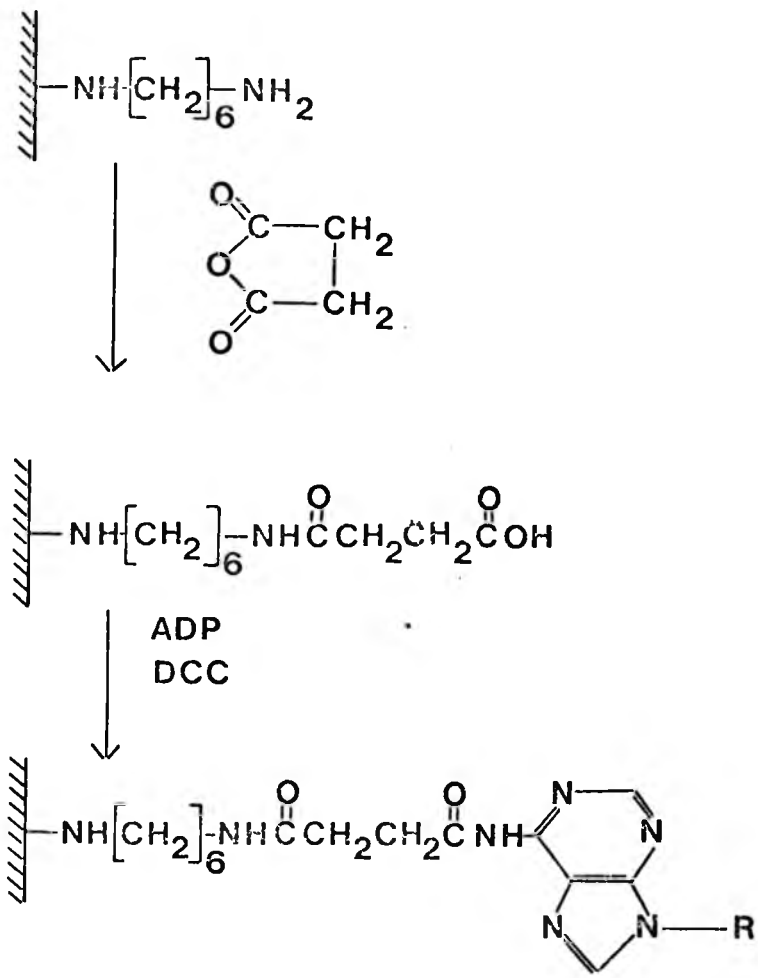
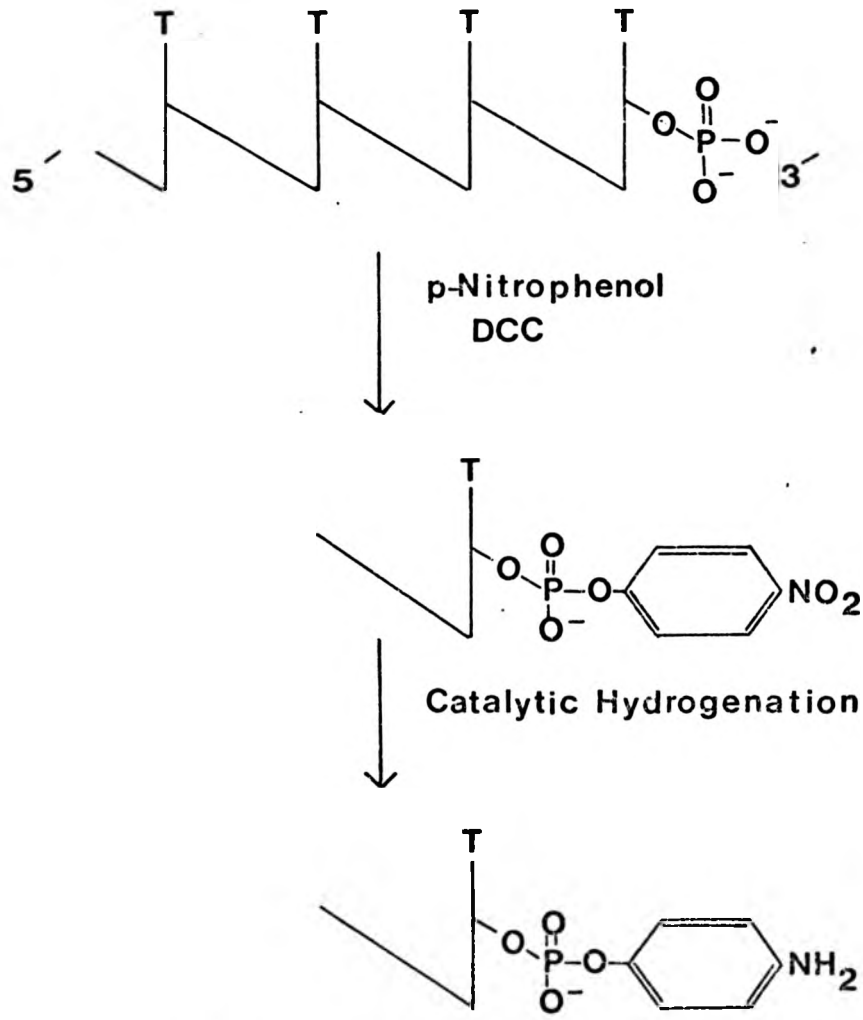

NH[CH2]6NH2
ADP
DCC
R

FIGURE 11

Preparation of p-aminophenyl oligo d(pT)


T
T
T
T
5′
3′
O
O
P
O⁻
O⁻
p-Nitrophenol
DCC
NO2
Catalytic Hydrogenation
NH2

is unsuitable for column chromatography. Methods for attaching polynucleotides directly to cyanogen bromide activated Sepharose have been described (Poonian, Schlaback and Weissbach, 1971), but the exact linkage of the polymer to the matrix is unknown and its stability is uncertain. Immobilized derivatives of poly(A) and poly(C) were prepared but did not retain polynucleotide phosphorylase. It was therefore decided to prepare an oligodeoxynucleotide ligand attached to the matrix through a spacer arm. It is known that the enzyme will bind very strongly to oligonucleotides and that the enzyme is competitively inhibited by deoxynucleotides (Godefroy, Cohn and Grunberg-Manago, 1970; Chou and Singer, 1971). Oligo d(pT) is readily synthesized by chemical polymerization giving a mixture of products of chain length n = 3 to n = 10. A ligand of this type was prepared (Figure 11).

Purification of polynucleotide phosphorylase by affinity chromatography

In a typical experiment, a column (1 x 18 cm) of oligo d(pT) Sepharose was equilibrated at room temperature with 0.10 M tris^{-HCl} 10 mM magnesium chloride, 1 mM EDTA, 2 mM mercaptoethanol, pH 8.2. A crude extract of B. stearothermophilus (20 ml, 36 u/ml) was applied to the column and washed on with starting buffer. The column was washed with the same buffer until no more protein was eluted. The buffer was then changed to a magnesium free buffer and a linear gradient of potassium chloride (0-2.0 M) was started. A peak of activity eluted at 1.2 M potassium chloride and had a specific activity of 1350 u/mg, representing an overall 900 fold purification. The elution profile is shown in Figure 12 and the results of a typical purification are given in Table 4. If the binding of the enzyme to the ligand is truly biospecific, it should be possible to elute the enzyme with a pulse of substrate. This has been achieved using either 1 mM ADP or 1 mM poly(A). However, this is prohibitively expensive on a preparative scale and presents a further purification step in the removal of substrate from the enzyme sample.

Effect of pH and temperature on the binding of the enzyme to the matrix

The results of experiments with the enzymes from E. Coli and B. stearothermophilus are shown in Figures 13 and 14. The binding

TABLE 4

Purification of B. Stearothermophilus Polynucleotide Phosphorylase

Step	Specific Activity	Purification	Total Units**
Cell lysate	1.50	1.00	6700
Polyethyleneimine	5.50	3.70	5360
Sepharose - 4B	10.00	6.70	5000
Oligo(dpT)-Sepharose	1350.00*	900	4000

* peak fraction, enzyme was purified on affinity column in batches of 20 ml

** 100 g cells (wet weight) were used.

FIGURE 12

Purification of polynucleotide phosphorylase from B. Stearothermophilus by affinity chromatography.

A solution of enzyme (20 ml, 3.6 mg protein/ml) was applied to a column (1.0 x 18.0 cm) of p-aminophenyl oligo d(pT) - Sepharose which had been equilibrated with 0.10M tris-HCl, 10 mM magnesium chloride, 1 mM EDTA, pH 8.2 at 20°. After elution of unbound protein, the enzyme was eluted with a gradient of potassium chloride (0-2.0 M) in 0.10 M tris-HCl, pH 8.2.

—— □ —— □—— Protein (mg/ml)

—— ▲ —— △—— Activity (units/mg)

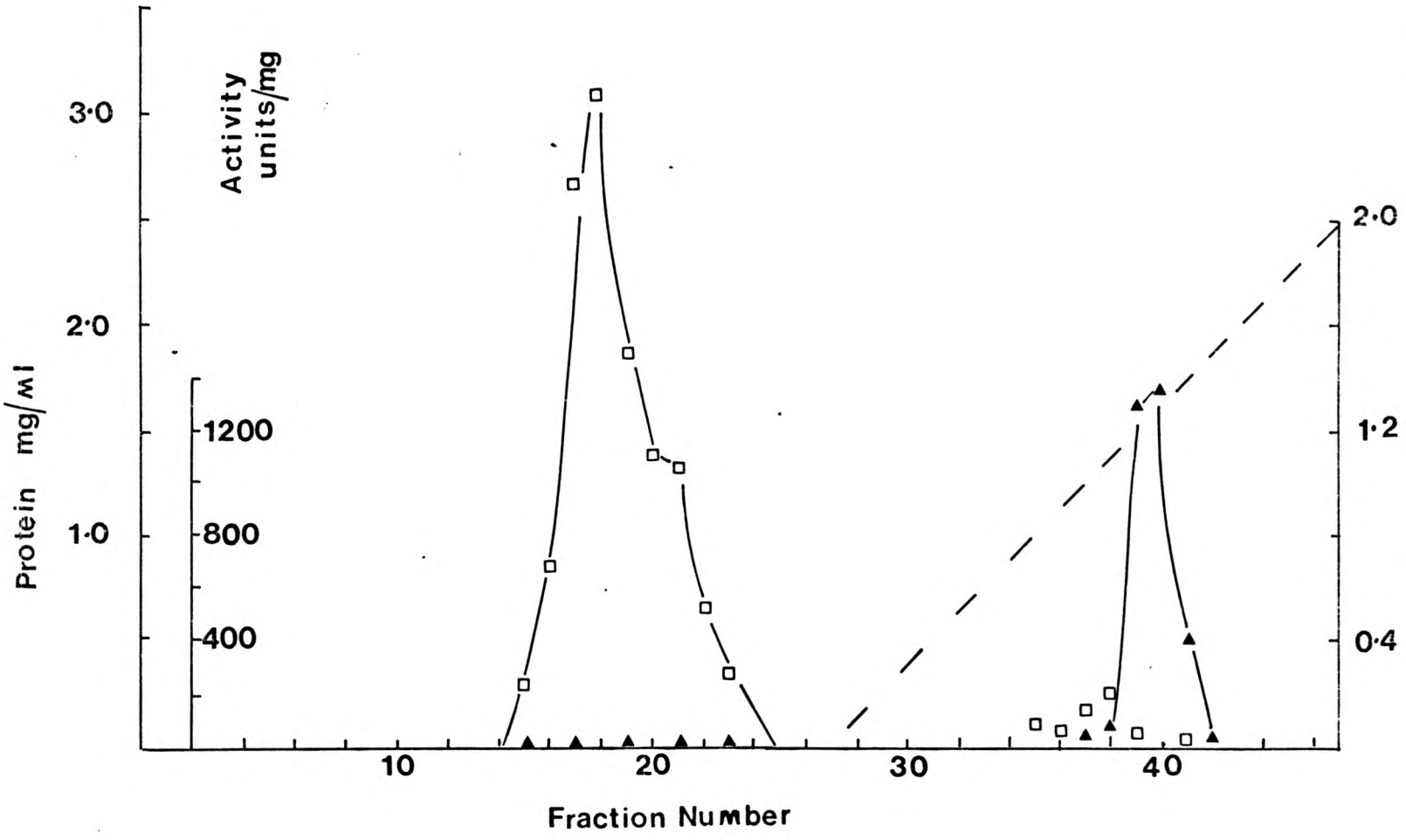

Protein mg/ml
3·0
2·0
1·0
Activity units/mg
1200
800
400
2·0
1·2
0·4
10
20
30
40
Fraction Number

FIGURE 13

Effect of pH on the binding of polynucleotide phosphorylase to the immobilized ligand.

——— ■ ——— Polynucleotide phosphorylase from E. Coli. Aliquots of enzyme (200 µl, 4.2 units) were added to the conjugate (2.5 ml) suspended in an equal volume of 0.10 M tris-HCl containing 10 mM magnesium chloride and 1 mM EDTA. The temperature was maintained at 10°. After fifteen minutes, a sample was taken and conjugate removed by filtration. The filtrate was then assayed for enzymic activity.

——— □ ——— Polynucleotide phosphorylase from B. Stearothermophilus. Aliquots of the enzyme (200µl, 11.5 units) were added to the conjugate (2.5 ml) suspended in an equal volume of 0.10 M tris-HCl containing 10 mM magnesium chloride and 1 mM EDTA. The temperature was maintained at 10°. After fifteen minutes, a sample was taken and the conjugate removed by filtration. The filtrate was then assayed for enzymic activity.

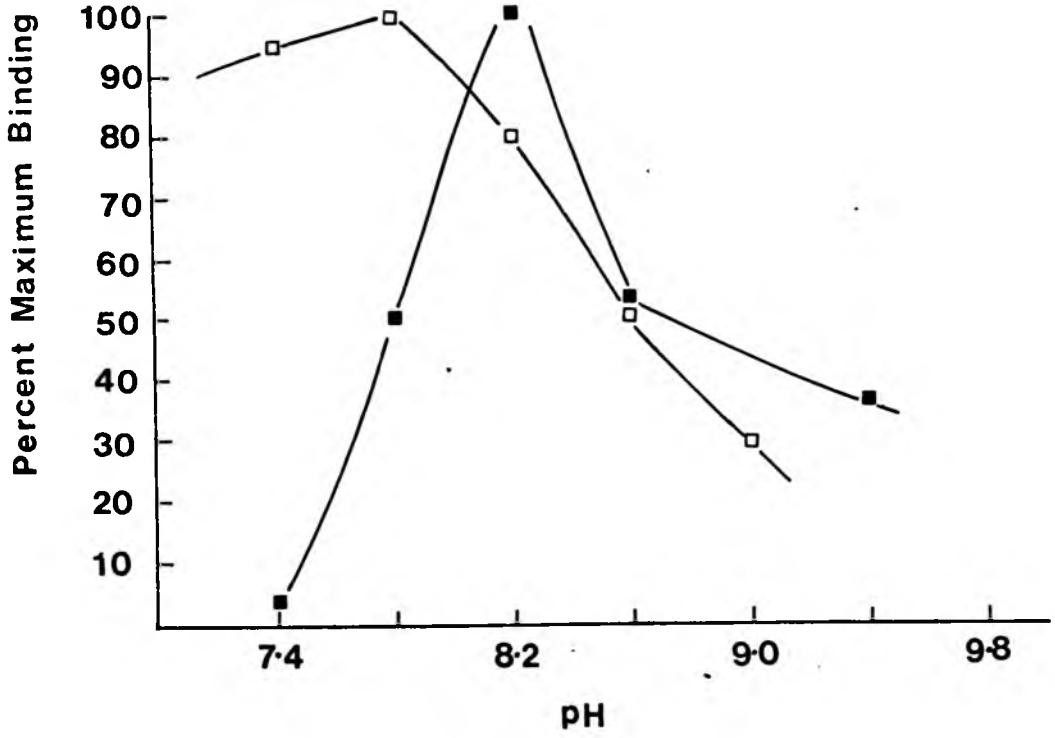

Percent Maximum Binding
100
90
80
70
60
50
40
30
20
10
7·4
8·2
9·0
9·8
pH

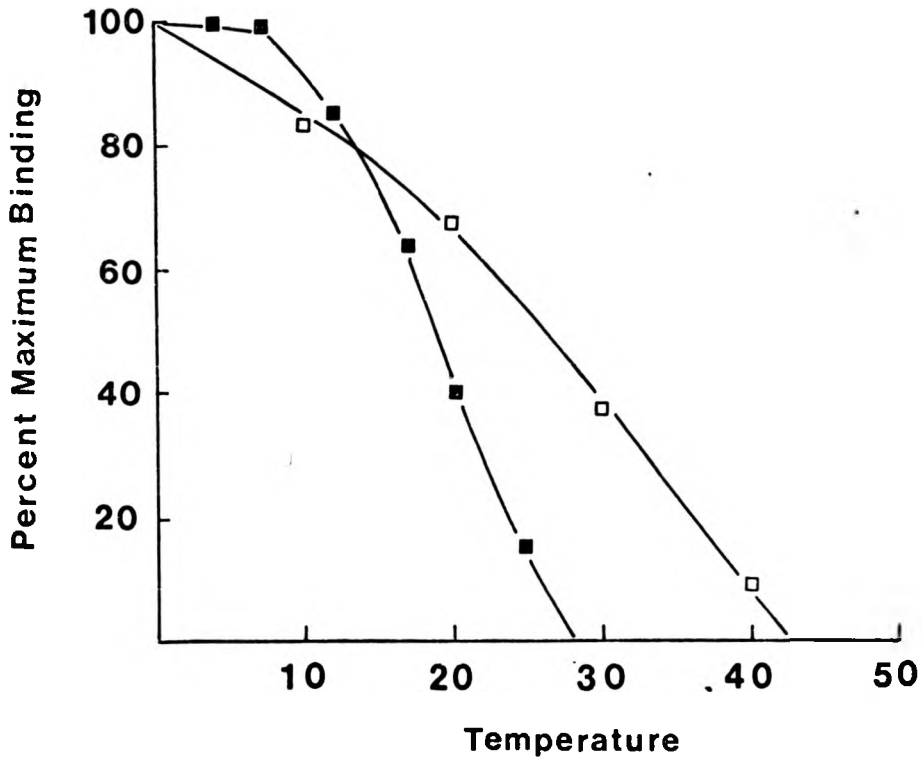
FIGURE 14

Effect of temperature on the binding of polynucleotide phosphorylase to the immobilized ligand.

Polynucleotide phosphorylase from E. Coli

—— ■ —— Aliquots of enzyme (200 µl, 4.2 units) were added to the conjugate (2.5 ml) suspended in an equal volume of 0.10 mM tris-HCl, containing 10 mM magnesium chloride and 1 mM EDTA, pH 8.2. After fifteen minutes, a sample was taken and the conjugate removed by filtration. The filtrate was then assayed for enzymic activity.

—— □ —— Polynucleotide phosphorylase from B. Stearothermophilus. Aliquots of the enzyme (200 µl, 11.5 units) were added to the conjugate (2.5 ml) suspended in an equal volume of 0.10 M tris-HCl containing 10 mM magnesium chloride and 1 mM EDTA, pH 7.8. After fifteen minutes, a sample was taken and the conjugate removed by filtration. The filtrate was then assayed for enzymic activity.


Percent Maximum Binding
100
80
60
40
20
10
20
30
40
50
Temperature

of the E. coli enzyme is strongest at pH 8.2, whilst the B. stearothermophilus enzyme binds strongly at pH 7.8. The strength of binding declines sharply on either side of the optimum.

The effect of temperature is less marked but the binding of the enzymes is strongest at temperatures below 10°. In the case of E. coli, the binding is 50% of maximum at 19° and the corresponding value for B. stearothermophilus enzyme is 27.5°. The results suggest that thermal elution could be a useful method of eluting the enzyme. The fact that binding is temperature dependent would also indicate that the binding of enzyme to ligand is not an ion-exchange effect but a biospecific event.

The capacity of the conjugate was determined under optimum pH and temperature conditions as 96 units/ml conjugate. In preparative work, an excess of ligand over enzyme was always used. A limitation of the ligand in preparative work is that nucleic acids and oligonucleotides must be removed before application of the crude enzyme to the column. It has been found that polyethyleneimine is the most effective agent for the precipitation of nucleic acids as the polynucleotide phosphorylase activity is precipitated at the same time. The enzyme activity can be extracted from the pellet affording a significant purification. There have been other examples of enzymes which have been coprecipitated with nucleic acids, for example, polynucleotide ligase and kinase activities can be separated when the kinase is precipitated with streptomycin sulphate (Panet et al., 1973). When nucleic acids were removed from crude preparations of polynucleotide phosphorylase by passage through a column of DEAE-cellulose (Thanassie and Singer, 1966), it was found that the enzyme was not effectively retained by the affinity column. This was probably because of competition from small oligonucleotides.

The oligo d(pT) used in this work was prepared by chemical polymerization which results in a product which is heterogeneous with respect to chain length. A more homogeneous product could have been obtained by the use of terminal transferase (Bollum, 1960) but the material would then have been available in much smaller quantities.

Purification of B. stearothermophilus polynucleotide phosphorylase by substrate elution from CM-Sephadex

A sample of the enzyme (2 ml, 10 u/ml) was dialysed against 5 mM MOPS, 1 mM EDTA, 2 mM 2-mercaptoethanol, pH 6.5 at 5° and applied to a column of CM-Sephadex C-50 which had been equilibrated with the same buffer. The column was washed with buffer until no more protein was eluted. No enzymic activity was eluted during this buffer wash. The column was then washed with buffer containing 1 mM ADP and fractions eluted with ADP showed enzyme activity Figure 15. 80% of the applied units were recovered and the specific activity was raised from 2.0 units/mg to 6.1 units/mg. A disadvantage of the procedure is the fact that the enzyme loses 50% of its activity during the dialysis step. This inactivation could be caused by the low pH during the dialysis step.

Properties of immobilized polynucleotide phosphorylase

Optimum conditions for binding polynucleotide phosphorylase to cyanogen bromide activated polysaccharides

Hoffman et al. (1970) have reported the preparation of immobilized polynucleotide phosphorylase using cyanogen bromide activated cellulose as a support. Tris buffer was used as the coupling buffer and it is probable that the amino group of the buffer reacted with the activated cellulose in competition with the enzyme, leading to a reduced loading of enzyme on the matrix. The effect of replacing tris with buffers lacking a reactive amino group was studied. The enzyme was dialysed against 0.01 M 2, 4, 6-collidine, pH 8.2 and then coupled to activated cellulose. The protein content of the conjugate was compared with that of a derivative prepared under identical conditions using tris buffer. The conjugate prepared in 2, 4, 6-collidine buffer contained 121 mg protein/g dry weight while the conjugate prepared in tris contained 43 mg protein/g dry weight. Collidine could be replaced by 0.10 M sodium bicarbonate, pH 8.0 or by 0.10 M sodium borate, pH 8.5.

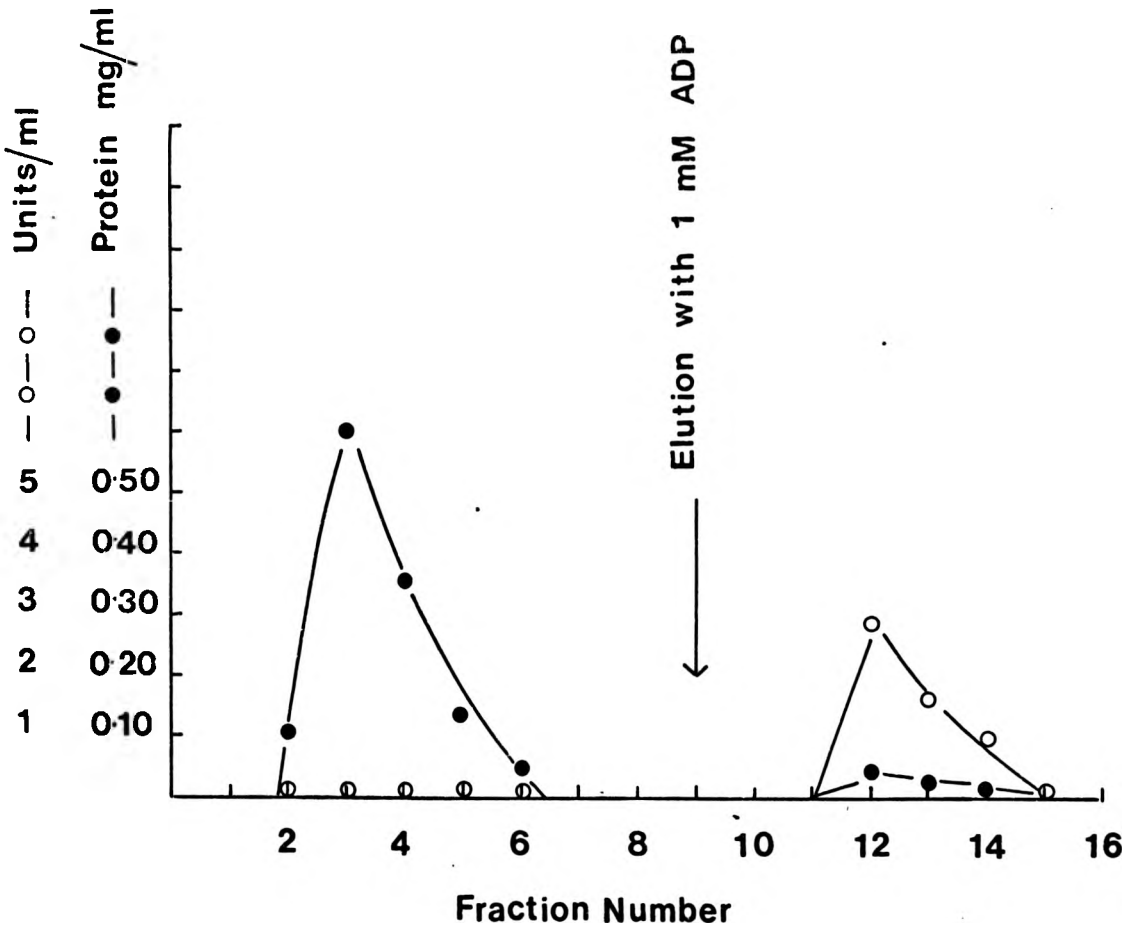
The effect of protein concentration on coupling yields and the final activity of the derivatives was then studied. Two support materials were used, Sephadex G-50 and Sepharose-4B, in order to compare the properties of conjugate where the enzyme was on the surface rather than in the pores of the matrix. Sephadex C-50 and Sepharose-4B were activated

FIGURE 15

Purification of polynucleotide phosphorylase from B. Stearothermophilus by substrate elution from CM-Sephadex. A column of CM-Sephadex C-50 (9.0 x 0.5 cm) was equilibrated with 5 mM MOPS, pH 6.5 containing 1 mM mercaptoethanol and 1 mM EDTA. A sample of enzyme (2.0 ml, 10 units/ml) was dialysed against the same buffer, applied to the column and washed through the column with starting buffer. After elution of unbound protein, the column was perfused with buffer containing 1 mM ADP. Fractions (2.0 ml) were collected and assayed for protein and enzymic activity.

—●—●— Protein (mg/ml)

—○—○— Activity (units/ml)


Units/ml
Protein mg/ml
5
4
3
2
1
0·50
0·40
0·30
0·20
0·10
Elution with 1 mM ADP
2
4
6
8
10
12
14
16
Fraction Number

with cyanogen bromide and coupled with various amounts of enzyme. The final reaction mixtures were adjusted to a constant volume by addition of coupling buffer. After thorough washing, the derivatives were assayed for protein and enzymic activity. The results are given in Table 5.

It can be seen that activated Sepharose binds more protein than Sephadex G-50. Polynucleotide phosphorylase from M. luteus was used in these studies and has a molecular weight of 270,000 (Letendre and Singer, 1975). The enzyme will therefore be excluded from the pores of Sephadex G-50 and will be bound on the surface of the matrix bead. The interior pores of the Sepharose matrix will be available for binding and this could account for the higher binding observed. A fluorescence study of the binding of Leucine aminopeptidase to cyanogen bromide activated Sepharose and Sephadex has shown that the enzyme (molecular weight 360,000) was evenly distributed through the Sepharose beads but was excluded from the Sephadex beads (Lasch, Iwig and Hanson, 1972). Contradictory results have been obtained in an autoradiographic study of the binding of lactoperoxidase (molecular weight 76,000) to activated Sepharose (David, Chino and Reisfeld, 1974). Autoradiographs indicated that the protein was bound in a crosslinked shell on the surface of the Sepharose beads. However, the Sepharose had been activated for very much longer than usual and the longer activation time could have resulted in a more crosslinked matrix which excluded the protein.

When the activity of the derivatives was expressed in terms of the dry weight of matrix, the Sephadex derivatives showed little variation in activity with protein content. Sepharose conjugates, on the other hand, showed an almost linear relationship between activity and protein content, the activity increases as the protein content decreases. This effect is observed with both derivativesbut is most marked with the Sephadex derivatives. It was concluded that the most efficient use of enzyme was to immobilize a small quantity of the enzyme on the matrix rather than trying to achieve the highest possible loading. Measurement of the apparent catalytic constants of cellulose derivatives with differing protein contents confirmed these observations.

TABLE 5

Binding of *M. luteus* polynucleotide phosphorylase to cyanogen bromide activated Sephadex G-50 and Sepharose-4B

Sephadex G-50

	% available protein coupled	Protein mg/g matrix	Activity µmole/hour/g	µmole/hour/mg protein
1.	70	42.00	3.10	0.074
2.	60	18.00	3.00	0.167
3.	33	7.20	3.14	0.436
4.	4	0.30	3.74	12.47

Sepharose-4B

1.	78	58.4	6.74	0.115
2.	68	25.5	3.97	0.156
3.	41	7.73	3.77	0.488
4.	16	1.50	2.10	1.400

Protein Content (mg/g matrix)	kcat(app)/kcat(free enzyme)
43	0.241
121	0.136

The reduction in observed catalytic efficiency with high protein loading is probably due to steric hindrance. This has been observed with other immobilized enzymes. The activity of CM-cellulose trypsin derivatives decreased with increasing protein content (Levin et al., 1964) and lactoperoxidase-sepharose conjugates also became less efficient at high protein loadings (David and Reisfeld, 1974).

Stability of the immobilized enzyme to thermal denaturation and inactivation by urea

The thermal stability of the enzyme was studied both in the free state and when immobilized. Aliquots of the enzyme were incubated at various temperatures, rapidly cooled and then assayed under standard conditions for polymerization activity. The results obtained with the M. luteus enzyme are shown in Figure 16. Immobilization on DEAE-cellulose by the triazine method resulted in a marked increase in thermal stability. Similar results were obtained with the enzyme from E. coli. In this case, the enzyme was immobilized on Celite-560 by the glutaraldehyde method and the results are shown in Figure 17. In general, the enzyme from E. coli is more stable than the enzyme from M. luteus.

The stability of the enzyme to denaturation by urea was determined by incubating aliquots of the immobilized enzyme in 1.0 M urea at 37°. At given time intervals, aliquots were transferred to a jacketed vessel and assayed for polymerization activity at the same urea concentration. Two derivatives of the M. luteus enzyme were used, a Sepharose-4B conjugate with an activity of 3.77 μmole/hour/g and a protein content of 7.73 mg/g and a Sephadex G-50 conjugate with an activity of 3.10 μmole/hour/g and a protein content of 42 mg/g. The results are shown in Figure 18. The Sepharose derivative showed greater stability, retaining 70% of its activity after sixty minutes incubation. Under the same conditions, the Sephadex derivative only retained 25% of its original activity.

FIGURE 16

Thermal stability of polynucleotide phosphorylase from M. luteus covalently attached to DEAE-cellulose.

Polymerization assays were carried out as described in the methods section. Samples were incubated at the specified temperature and aliquots were taken, cooled and assayed at 30°.

—●—●— soluble enzyme, 20 minutes

—▲—▲— immobilized enzyme, 20 minutes

—□—□— immobilized enzyme, 60 minutes

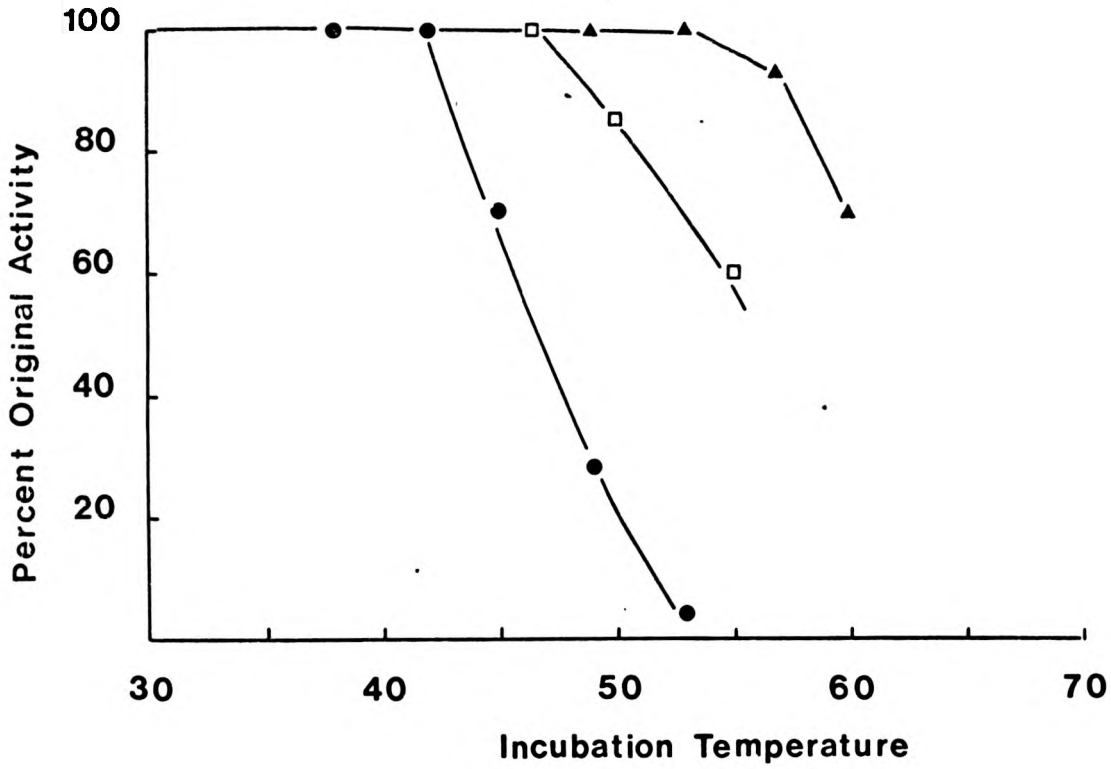

100
80
60
40
20
Percent Original Activity
30
40
50
60
70
Incubation Temperature

FIGURE 17

Thermal stability of polynucleotide phosphorylase from E. Coli covalently attached to Celite-560.

Polymerization assays were carried out as described in the methods section. Samples were incubated at the specified temperature for 30 minutes when aliquots were removed, cooled and assayed at 30°.

—— ■ —— □ —— immobilized enzyme

—— O —— O —— free enzyme

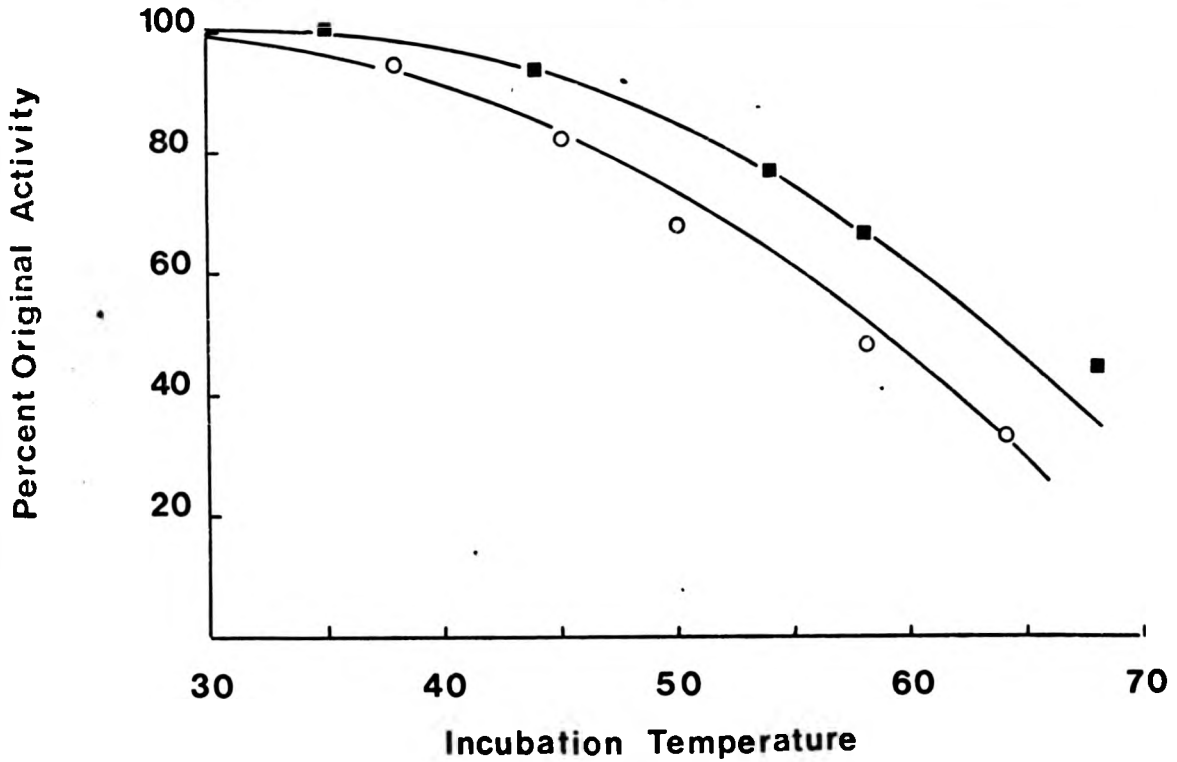

Percent Original Activity
100
80
60
40
20
30
40
50
60
70
Incubation Temperature

FIGURE 18

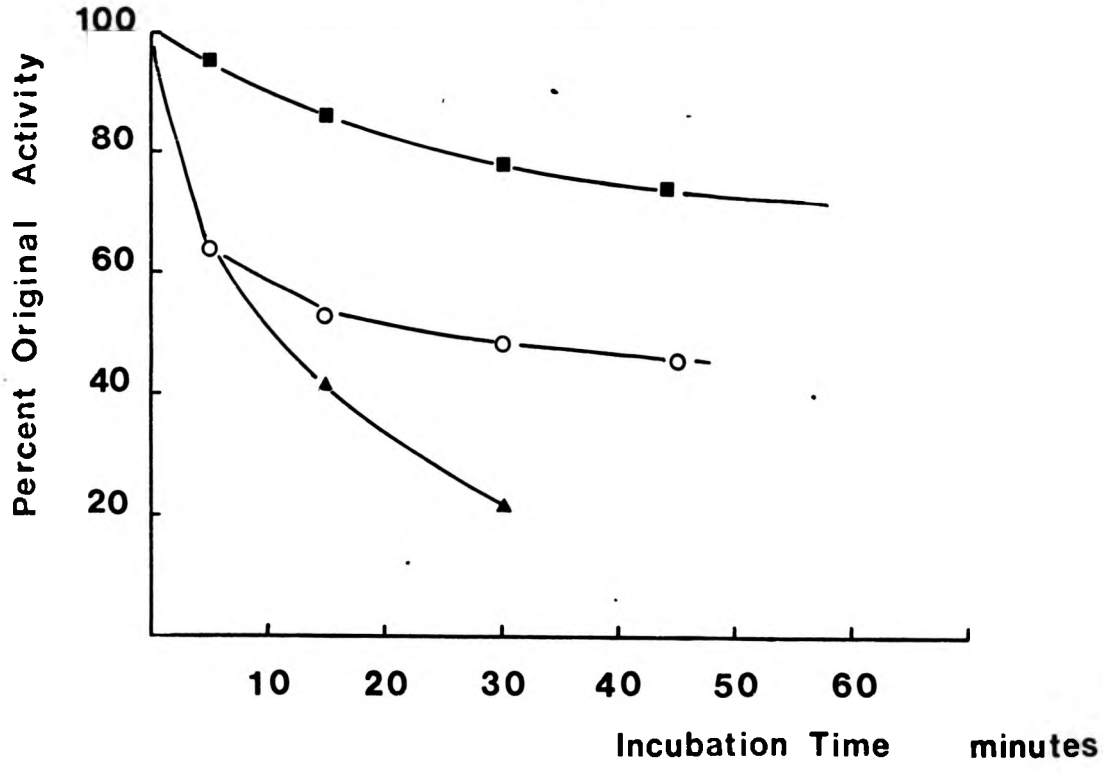
Stability of polynucleotide phosphorylase from M. luteus to urea denaturation.

Samples were incubated in 0.10 M-Tris pH 9.0 containing 1.0 M urea at 37°. Aliquots were removed and assayed for activity in polymerization buffer containing 1.0 M urea.

—— ■ —— □ —— Immobilized on Sepharose-4B

—— O —— O —— free solution

—— ▲ —— △ —— immobilized on Sephadex G-50


Percent Original Activity
100
80
60
40
20
10
20
30
40
50
60
Incubation Time
minutes

Kinetics

All the immobilized enzyme derivatives obeyed Michaelis Menton kinetics when assayed for polymerization in stirred suspension. The values of the Michaelis constants of some derivatives of the M. luteus enzyme are given in Table 6. The apparent values of the Michaelis constant are unchanged when the enzyme is covalently bound to cellulose. This is expected from previous work with charged substrates and enzymes attached to uncharged supports (Hornby, Lilly and Crook, 1967). The decrease in Km observed when the enzyme was attached to alkylamine glass could be attributed to the positive charge on the matrix leading to a higher substrate concentration in the micro-environment of the enzyme.

When the enzyme is attached to Sepharose, there is a four fold increase in the apparent value of Km. A theoretical model has been proposed to describe the kinetics of enzymes immobilized in spherical gel particles, such as Sepharose (Kasche et al., 1971). In this model, the rate of product formation is proportional to the square root of the enzyme concentration at low substrate concentrations and proportional to enzyme concentration at high substrate concentrations. The establishment of concentration gradients as the substrate diffuses through the pores of the gel will lead to an increase in the apparent value of Km. This could explain the observed increase in the Km although the enzyme cannot be severely limited by diffusion as Lineweaver Burk plots are linear.

A similar increase in Km is observed when the E. coli enzyme is bound to Sepharose. In this case, the apparent Km only increases from 1.25×10^{-4} M to 1.67×10^{-4} M. The protein content of the derivative was 430 mg/g. Again, no deviation from linearity was observed in the Lineweaver Burk plots. Another method of detecting diffusion limitations is to compare the activation energies of the immobilized enzyme and the free enzyme. The results obtained with E. coli polynucleotide phosphorylase in free solution and covalently bound to Sepharose are shown in Figure 19. There is a slight drop of 2% in the activation energy but this is probably not significant. Diffusional limitations would only be expected to occur with very active

TABLE 6

Kinetic constants of immobilized M. luteus polynucleotide phosphorylase

Support	Protein mg/g matrix	Km (app) M	kcat app/ kcat free
Free enzyme	-	1.0×10^{-4}	1.00
Cellulose (a)	43	1.0×10^{-4}	0.241
Cellulose (b)	121	1.0×10^{-4}	0.136
Sepharose-4B	475	4.0×10^{-4}	0.325
Porous glass	6.8	0.50×10^{-4}	0.704

(a) coupled in Tris buffer

(b) coupled in collidine buffer

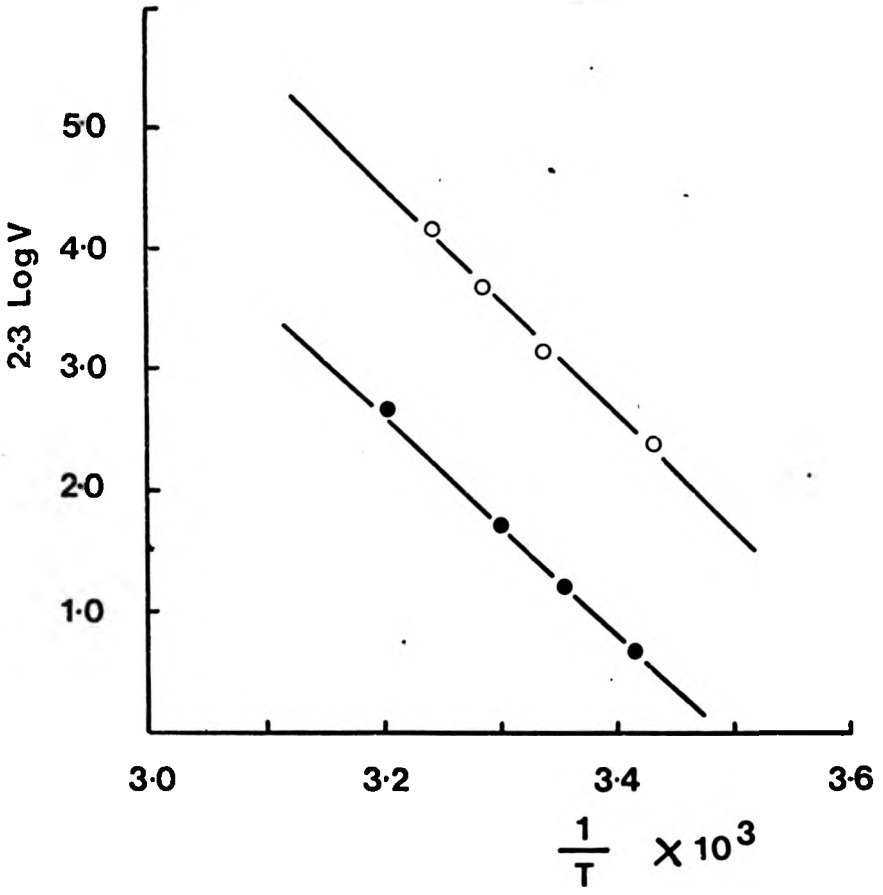
Assays carried out in stirred suspension as described in the Methods Section with ADP as substrate

FIGURE 19

Arrhenius plots for polynucleotide phosphorylase from E. coli in free solution and covalently bound to Sepharose 4B. Polymerization assay carried out as described in the Methods Section using 10 mM ADP as substrate.

—— O—— O—— free enzyme

—— ●—— ●—— immobilized


5·0
4·0
3·0
2·0
1·0
2·3 Log V
3·0
3·2
3·4
3·6
1/T X 10^3

enzyme preparations (Sundaram, Tweedale and Laidler, 1970). The activity of the polynucleotide phosphorylase used in these experiments is lower than that of enzyme systems for which diffusional limitations have been observed and it can be concluded that immobilized polynucleotide phosphorylase is not diffusion limited when assayed in stirred suspension.

A study of the effect of ionic strength on the kinetics of the immobilized enzyme was carried out using DEAE-cellulose as the supporting matrix. This would give a system in which the substrate and matrix have opposite charge. At low ionic strength the apparent Km should be lower than that of the free enzyme system (Wharton, Crook and Brocklehurst, 1968b). The M. luteus enzyme was successfully attached to DEAE-cellulose by the triazine method. The derivative used in these studies had a protein content of 33 mg/g and an activity of 98 μmole/hour/g. Lineweaver-Burk plots obtained with the free and immobilized enzyme are shown in Figures 20 and 21. The Km of the free enzyme is independent of ionic strength whereas the apparent Km of the immobilized enzyme is dependent on ionic strength and is decreased at low ionic strength. In addition to the effect on the apparent Km, there is a positive ionic strength effect on the value of kcat. A threefold increase in the ionic strength leads to a fivefold increase in the apparent kcat of the immobilized enzyme. A similar effect on kcat is observed with the soluble enzyme and would indicate that the substrate is interacting with a negatively charged group on the protein.

The effect of ionic strength on the hydrolysis of esters by bromelain covalently bound to CM-cellulose has been studied (Wharton, Crook and Brocklhurst, 1968b). An equation was derived which described the observed perturbation of the Michaelis constant.

$$\frac{I}{\text{app Km}} = \frac{I}{\gamma \text{app Km}_{(\text{lim})}} + \frac{Z\,mc}{2\ \text{app Km}_{(\text{lim})}} \qquad (1)$$

where I = ionic strength

app Km = apparent value of Michaelis constant

app $\text{Km}_{(\text{lim})}$ = apparent value of Michaelis constant when net charge on matrix = 0

FIGURE 20

Effect of ionic strength on kinetics of ADP polymerization by polynucleotide phosphorylase from M. luteus.

—□—□— Ionic strength = 0.0167

—●—●— Ionic strength = 0.0567

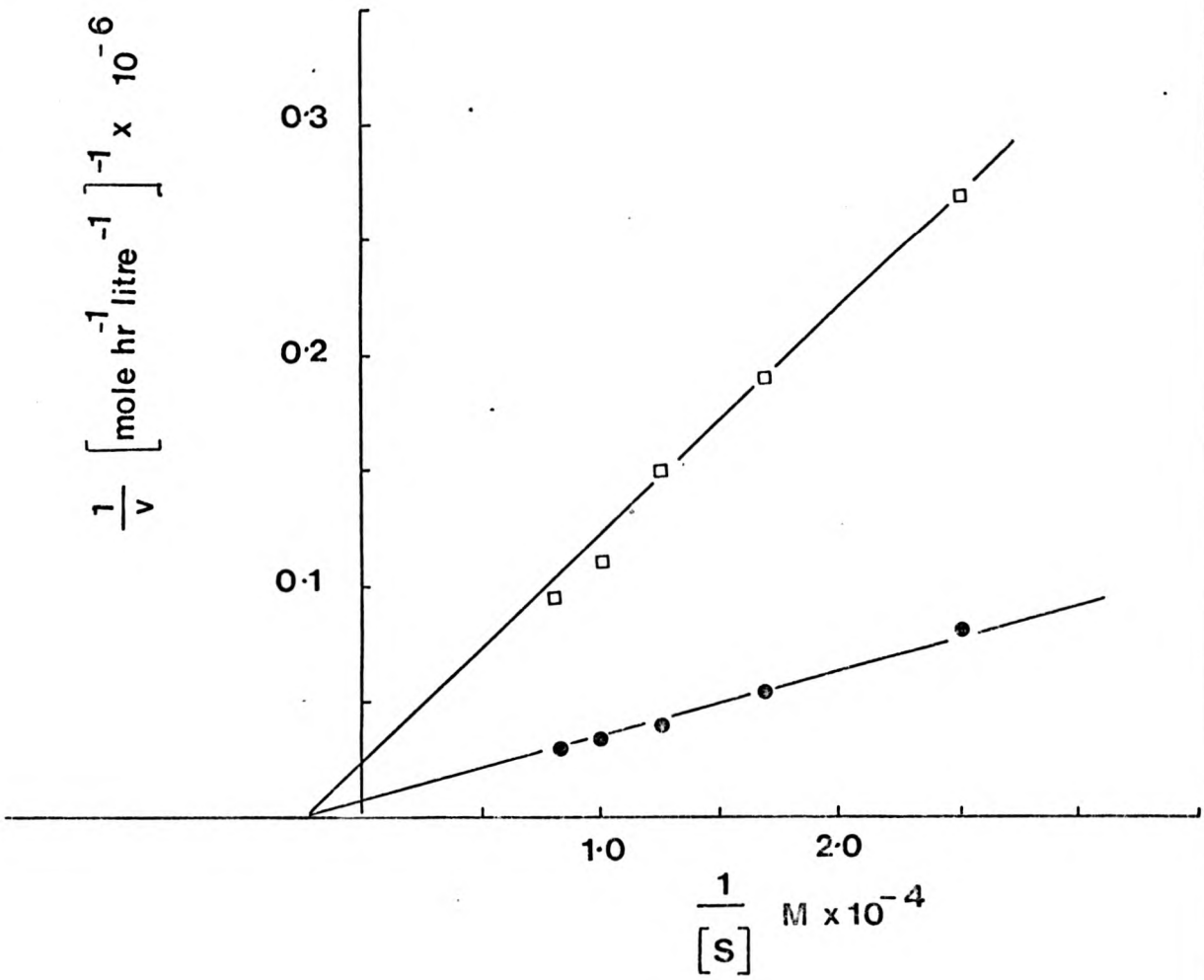
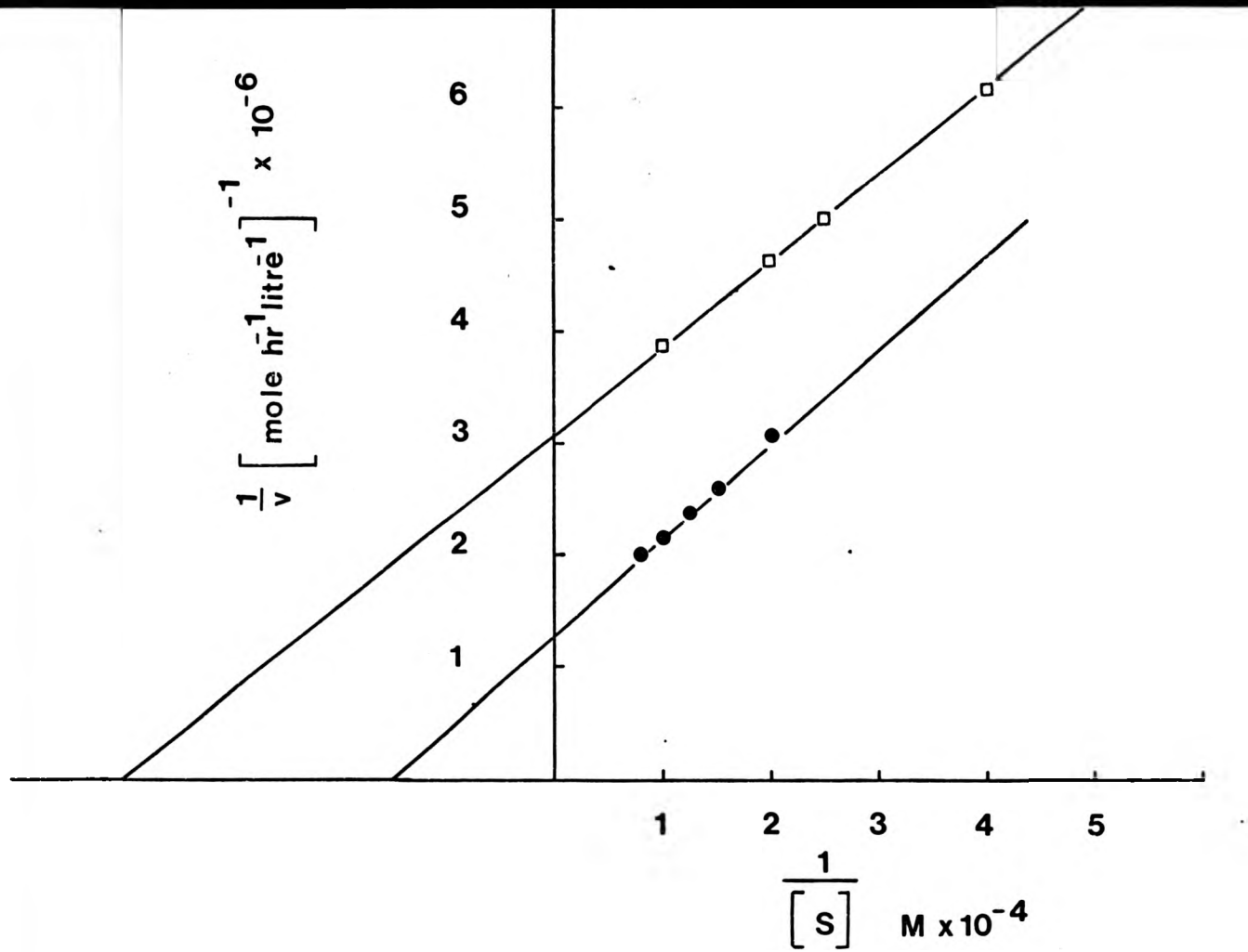

$\frac{1}{v}$ [mole hr^{-1} litre^{-1}]$^{-1}$ x 10^{-6}
0·3
0·2
0·1
1·0
2·0
$\frac{1}{[S]}$ M x 10^{-4}

FIGURE 21

Effect of ionic strength on kinetics of ADP polymerization by polynucleotide phosphorylase from M. luteus covalently attached to DEAE-cellulose.

—□—□— ionic strength = 0.0567

—○—○— ionic strength = 0.1567


$\frac{1}{v}\left[\text{mole hr}^{-1}\text{litre}^{-1}\right]^{-1} \times 10^{-6}$
6
5
4
3
2
1
1
2
3
4
5
$\frac{1}{[S]}$ M x 10^{-4}

γ = ratio of the mean ion activity coefficients of the matrix and bulk phases.

z = modulus of the number of charges on the matrix.

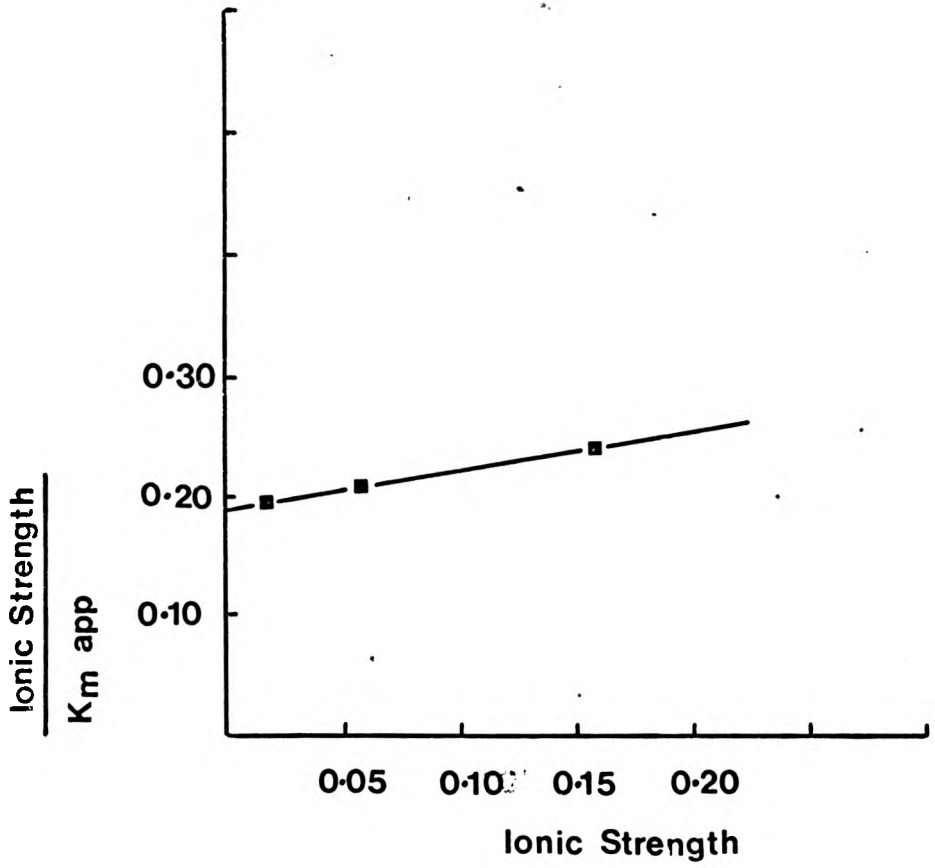
mc = concentration of matrix in its hydrated form.

A plot of $1/_{app\ Km}$ against I is shown in Figure 22 and is linear as predicted from Theory. If polynucleotide phosphorylase was attached to CM-cellulose, the apparent value of Km would be expected to increase. Attempts were made to couple the enzyme to CM-cellulose by the acid azide method (Mitz and Summaria, 1961) but it was not possible to prepare an active derivative by this method. The DEAE-cellulose conjugate was not suitable for the prepartion of polymers as the product binds strongly to the ion-exchange groups of the matrix and is only released on washing with strong salt solutions. This is impractical when it is desired to use the conjugate in a continuous process.

It is of interest to establish whether the kinetic mechanism of an enzyme is altered on immobilization. One approach to this problem is to study the effect of inhibitors on the enzyme in its free and immobilized forms (Preuveneers et al., 1973). Sodium thiophosphate was found to be an inhibitor of the enzyme when the polymerization of thiophosphate analogues of nucleoside diphosphates was being studied. Further studies were then made to establish the type of inhibition caused by sodium thiophosphate and a comparison was made between the free and immobilized enzyme. The inhibitor causes non-competitive inhibition of ADP polymerization by the M. luteus enzyme and similar results were obtained with the enzyme from E. coli (Figure 23). In this form of inhibition, the inhibitor can combine with the enzyme or with the enzyme-substrate complex. The enzyme-inhibitor-substrate complex does not react to form product or does so only at a slower rate than the enzyme-substrate complex (Gutfreund, 1965). If it is proposed that there is a specific substrate binding site and a catalytic site controlling the reaction of substrate to form product, a non-competitive inhibitor could bind to the catalytic site, changing the reactivity without changing the substrate specificity. Once the inhibition pattern of the soluble enzyme had been established, the properties of the immobilized

FIGURE 22

Dependence upon ionic strength of Km (app) for the DEAE-cellulose-polynucleotide phosphorylase catalysed polymerization of ADP.


Ionic Strength / Km app
0·30
0·20
0·10
0·05
0·10
0·15
0·20
Ionic Strength

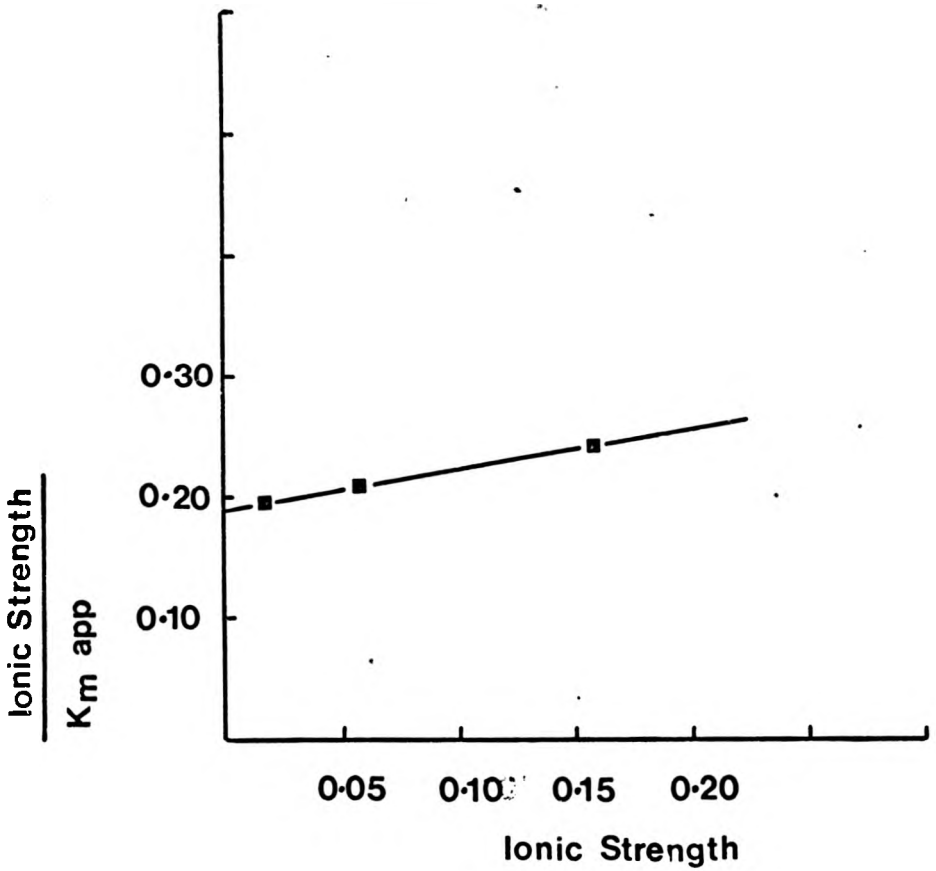
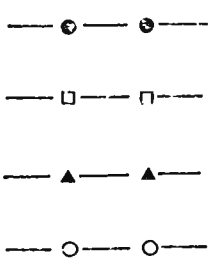
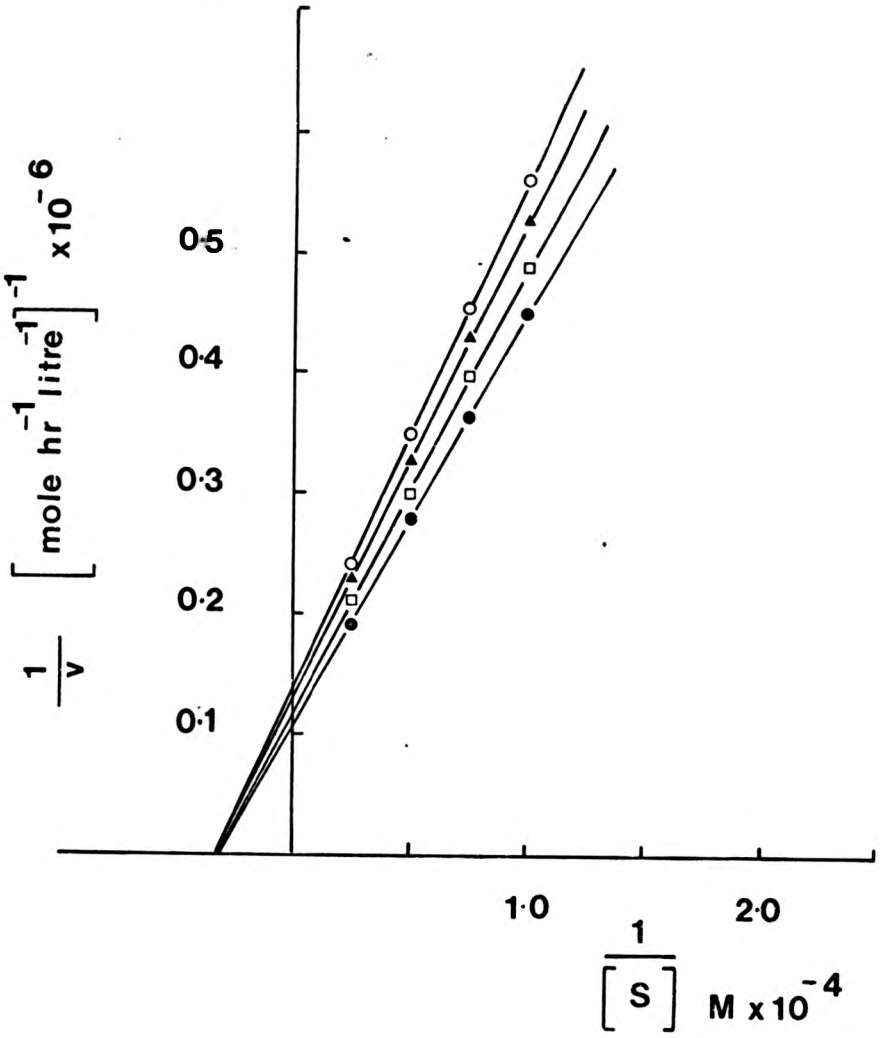

Ionic Strength / Km app
0·30
0·20
0·10
0·05
0·10
0·15
0·20
Ionic Strength

FIGURE 23

ADP polymerization by polynucleotide phosphorylase from E. coli.

Noncompetitive inhibition by sodium thiophosphate.




$\frac{1}{v}$ $\left[\text{mole hr}^{-1}\text{ litre}^{-1}\right]^{-1}$ $\times 10^{-6}$
0·5
0·4
0·3
0·2
0·1
1·0
2·0
$\frac{1}{[S]}$ M $\times 10^{-4}$

enzyme were studied. A conjugate of E. coli polynucleotide phosphorylase bound to Sepharose-4B was used in these studies. The inhibition was shown to be non-competitive indicating that there is no significant change in the mechanism of the enzyme after immobilization (Figure 24). The inhibitor constants were determined from plots of the reciprocal initial velocity against inhibitor concentration at various substrate concentrations. The inhibitor constant for the immobilized enzyme is 7 μM while the value for the soluble enzyme is 14 μM. Further evidence for the existence of two binding sites on the enzyme has been obtained from studies of the polymerization of 8 Bromo ADP which acts as a non-competitive inhibitor of ADP polymerization (Ikehara, Tazawa and Fukui, 1969; Ikehara and Fukui, 1973).

Synthesis of polynucleotides by immobilized polynucleotide phosphorylase

One of the main objectives of the study of immobilized polynucleotide phosphorylase was to prepare a derivative of the enzyme which could be used repeatedly for the synthesis of polynucleotides. In particular, it was hoped that the use of a re-usable catalyst would simplify the polymerization of atypical substrates by allowing the use of large quantities of enzyme.

Polynucleotide phosphorylase synthesizes a polynucleotide in a processive manner, i.e. a polymer is not released from the enzyme until it is completed. Similarily, if a polymer is being degraded, the degraded molecule will not be released until short oligonucleotides are formed (Chou and Singer, 1970 a, b). It is therefore important to minimize phosphorolysis by selecting conditions where polymerization is favoured. One of the most critical parameters is pH. The pH optimum for the polymerization reaction with the soluble enzyme is 9.0 while the optimum for the phosphorolysis reaction is 8.0. On immobilization, the pH optima for the reactions are altered. The optimum for polymerization is raised to a more alkaline value while the pH optimum for phosphorolysis is unchanged or slightly lowered. The pH profiles of M. luteus polynucleotide phosphorylase in free solution and immobilized on cellulose are shown in Figure 25. The pH optima of other enzyme derivatives are presented in Table 7. The greatest separation of the pH optima is observed with the cellulose derivatives.

FIGURE 24

ADP polymerization by polynucleotide phosphorylase from E. coli, immobilized on Sepharose 4B. Noncompetitive inhibition by sodium thiophosphate.

—○—○— no thiophosphate

—▲—▲— 1 μM

—□—□— 2.5 μM

—●—●— 5.0 μM

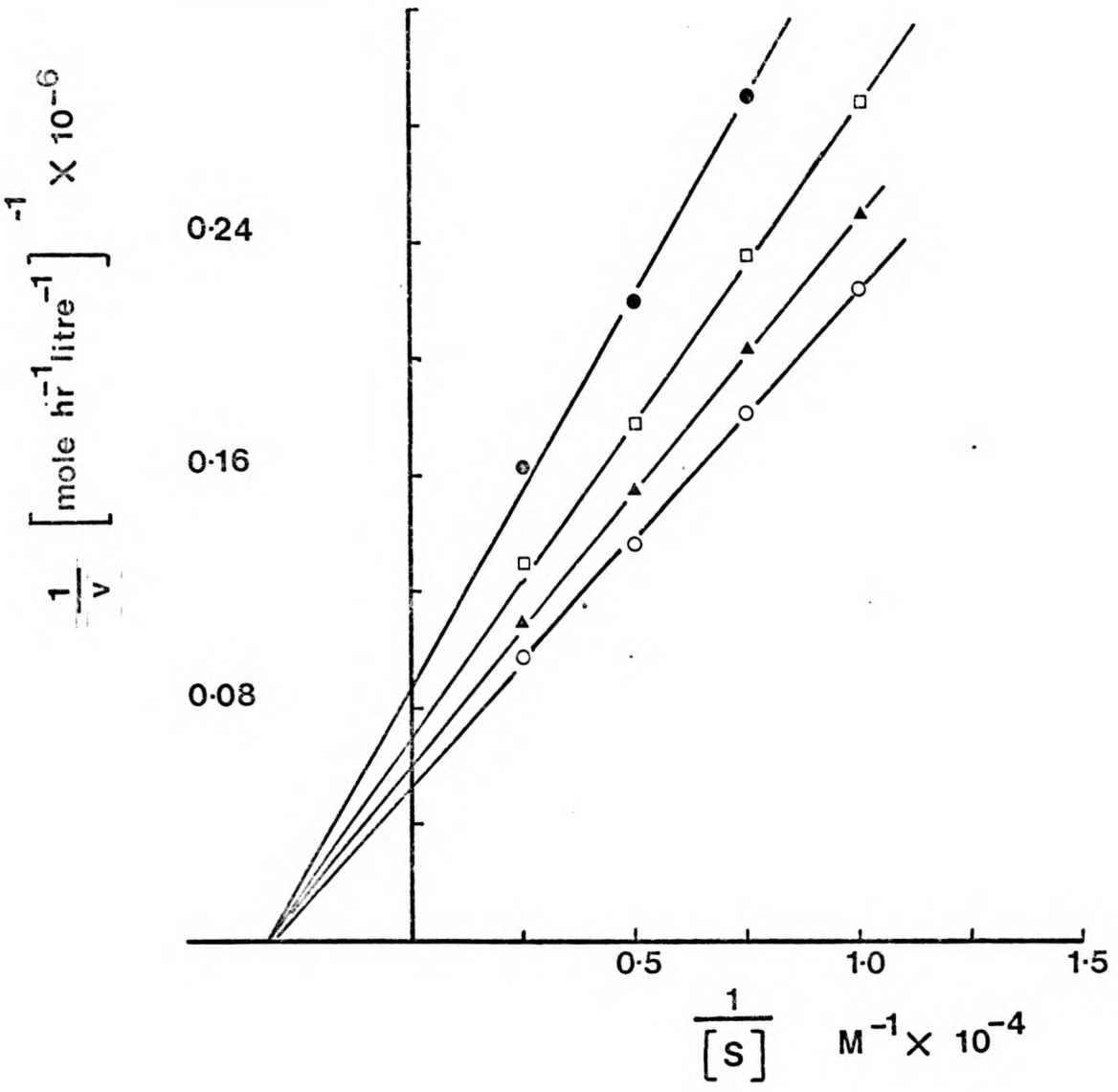

$\frac{1}{v}$ $\left[\text{mole hr}^{-1}\text{litre}^{-1}\right]^{-1} \times 10^{-6}$
0·24
0·16
0·08
0·5
1·0
1·5
$\frac{1}{[S]}$ $M^{-1} \times 10^{-4}$

TABLE 7

pH optima of polynucleotide phosphorylase

Enzyme Derivative	pH optima	
	phosphorolysis *	polymerization **
M. luteus		
free	8.10	9.00
cellulose[1]	7.70	9.25
sepharose[1]	8.00	9.25
porous glass[2]	8.50	9.40
E. Coli		
free	7.80	9.00
sepharose	7.80	9.25

* Poly(A) as substrate

** ADP as substrate

1 Cyanogen bromide activation of matrix

2 Cross linking by glutaraldehyde to aminoalkyl glass.

Changes in the pH optima of enzyme reactions after immobilization have been observed previously and are most often observed with hydrolytic enzymes such as trypsin. Polyanionic derivatives of trypsin show a rise in pH optima while in polycationic derivatives there is a decrease in the pH optimum (Goldstein, Levin and Katchalski, 1964). These effects have been ascribed to a change in the pH of the microenvironment of the enzyme however it is difficult to explain the observed changes in optima for the polynucleotide phosphorylase derivatives in terms of a change in local pH. The most pronounced effects are seen with the cellulose derivatives and the effect could be due to the presence of charged groups which are known to be present as impurities in cellulose and other polysaccharides (Porath, Janson and Laas, 1971). The separation of pH optima for the forward and reverse reactions has important synthetic applications as degradation of polymer is minimized.

The immobilized enzyme was operated in the form of a packed bed and the synthesis of poly(A), poly(C) and poly(Cl^5C) was studied. The effect of flow rate on conversion of substrate was measured and the results are given in Figure 26. The effect of input substrate concentration is shown in Figure 27. It can be seen that the conversion rate is inversely proportional to the flow rate and the initial substrate concentration.

The kinetics of immobilized enzymes in packed beds have been studied in detail by Lilly, Hornby and Crook (1966). If it is assumed that the enzyme follows Michaelis Menten kinetics and that the reaction reaches a steady state, the following equation describes the behaviour of an enzyme in a packed bed.

$$PSo = \text{app Km ln } (1-P) + {}^{C}/_{Q} \quad \text{(2)}$$

where P = percentage conversion

So = initial substrate concentration.

C = reaction capacity (mole min^{-1})

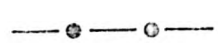
Q = flow rate (ml min^{-1})

app Km = apparent value of Michaelis constant (M).

A plot of P.So against ln (1-P) should give a straight line with slope equal to the app Km. The results obtained with a packed bed of

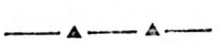
FIGURE 25

pH profiles for polymerization and phosphorolysis reactions catalysed by polynucleotide phosphorylase from *M. luteus*.



polymerization free enzyme

polymerization immobilized on cellulose.



phosphorolysis free enzyme

phosphorolysis immobilized on cellulose.

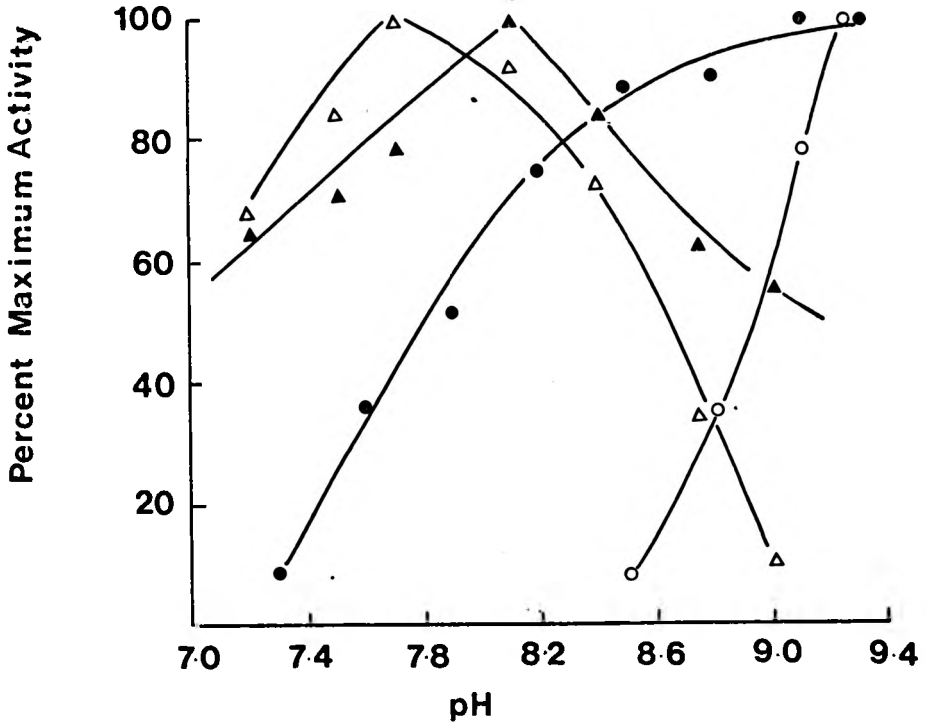

Percent Maximum Activity
100
80
60
40
20
7·0
7·4
7·8
8·2
8·6
9·0
9·4
pH

FIGURE 26

Effect of flow rate on the polymerization of ADP by a column (5.5 x 0.9 cm) containing polynucleotide phosphorylase from *E. coli* immobilized on celite-560.

—■—■— 0.50×10^{-4} M ADP

—○—○— 1.00×10^{-4} M ADP

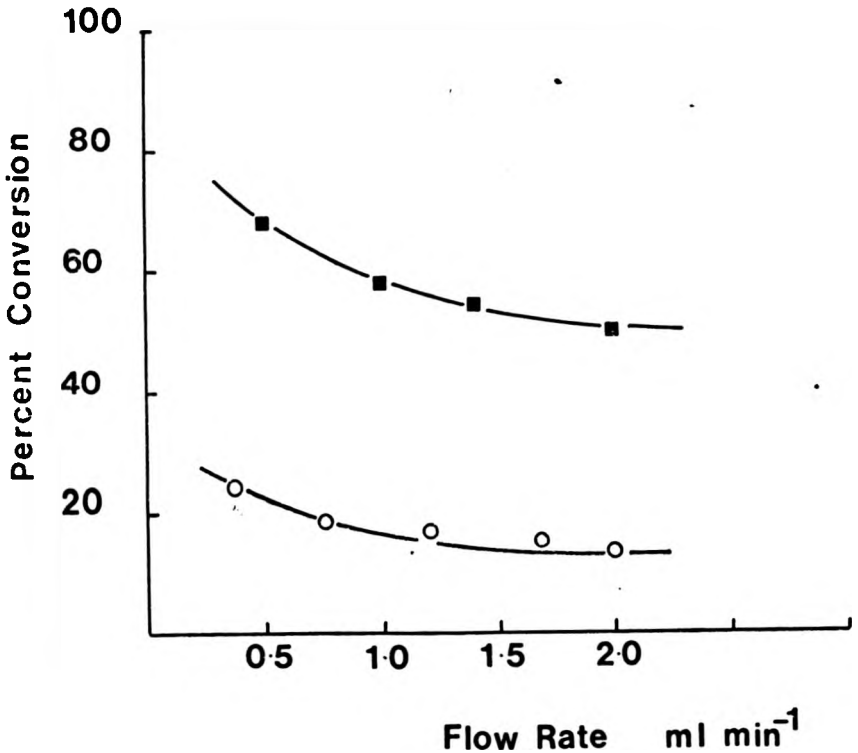

100
80
60
40
20
Percent Conversion
0·5
1·0
1·5
2·0
Flow Rate ml min^{-1}

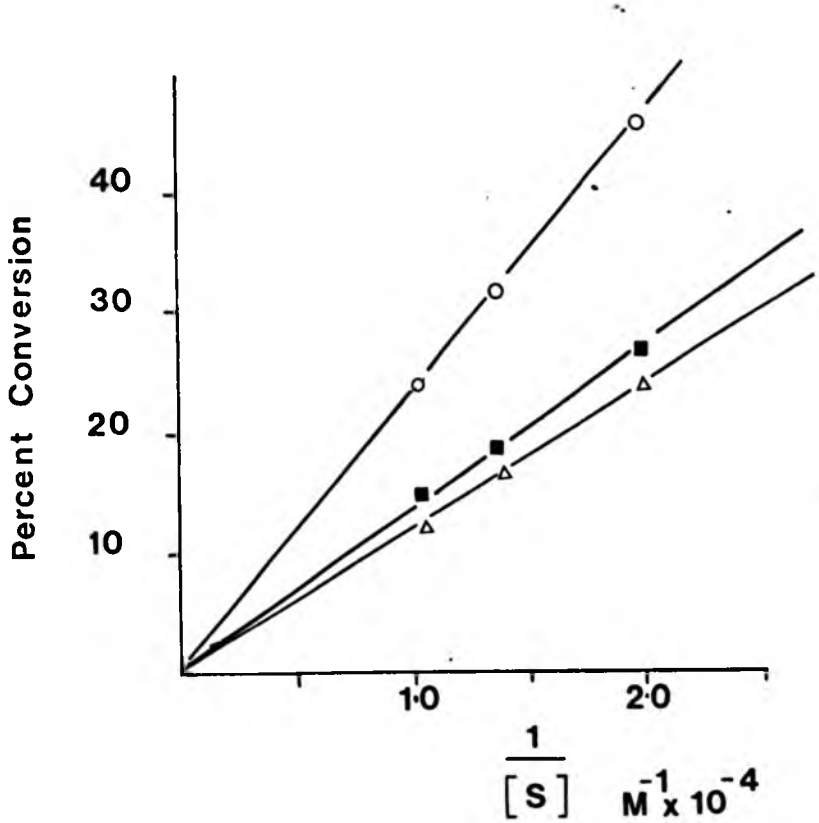
FIGURE 27

Polymerization of atypical substrates by a packed bed (10.8 x 0.9 cm) of polynucleotide phosphorylase from E. coli immobilized on Sepharose-4B. Flow rate = 0.50 ml min^{-1}.

—O—O— 1.0×10^{-4} M ADP

—□—□— 1.0×10^{-4} M CDP

—△—△— 1.0×10^{-4} M Cl^5CDP


Percent Conversion
40
30
20
10
1·0
2·0
1/[S] M⁻¹ x 10⁻⁴

E. coli polynucleotide phosphorylase bound to Celite-560 are shown in Figure 28. The derivative had a protein content of 14.3 mg/g and in stirred suspension, the apparent Km for ADP polymerization was 3.0×10^{-4}M. It can be seen that there is significant deviation from the behaviour predicted in Equation 2. The validity of Equation 2 depends upon there being piston flow through the packed bed, i.e. all elements of the substrate solution move through the bed at equal velocities and there is no mixing. This condition can be checked by preparing an F diagram (Lilly, Hornby and Crook, 1966). A solution of ADP was passed through the packed bed and the appearance of ADP in the effluent was monitored spectrophotometrically at 260 nm. The void volume of the packed bed was determined and the holdback, H, was calculated from the ratio of the area under the curve between v = o and v = void volume to the total area between these limits and the zero and maximal values of A260. For a column with a total volume of 4.3 ml, the void volume was 3.2 ml and the holdback was 0.0624 (Figure 29). Under ideal conditions, H should equal zero but the observed value of H is not great enough to account for the observed deviations in this case.

At the inlet of a column of polynucleotide phosphorylase, de novo synthesis of polymer will take place and as completed polymer is released from the enzyme, it will move down the column. Further down the column, the polymer could be utilized as primer and there would then be two types of polymer formation taking place - de novo synthesis and incorporation into primer. The rate of primed and unprimed reaction would be different and the steady state assumption would no longer be valid. At low flow rates, there will be a greater conversion of substrate into polymer and the predominant species passing through the column will be polymer. If there is a high concentration of polymer and groups, chain elongation will be restricted to the addition of a small number of nucleotide residues.

The behaviour of another phosphorylase enzyme has been studied after immobilization on porous glass (Marshall and Walter, 1972). The enzyme glycogen phosphorylase, catalyses the reaction

$$x \text{ D-glucosyl phosphate} + (\text{D-glucose})_n \longrightarrow (\text{D-glucose})_{n+x} + x\text{Pi}$$

FIGURE 28

Kinetics of polymerization of ADP catalysed by a column (5.5 × 0.90 cm) of polynucleotide phosphorylase from E. coli immobilized on celite-560.

—— ●—— ●—— flow rate = 1.60 ml min^{-1}

—— □—— □—— flow rate = 1.00 ml min^{-1}

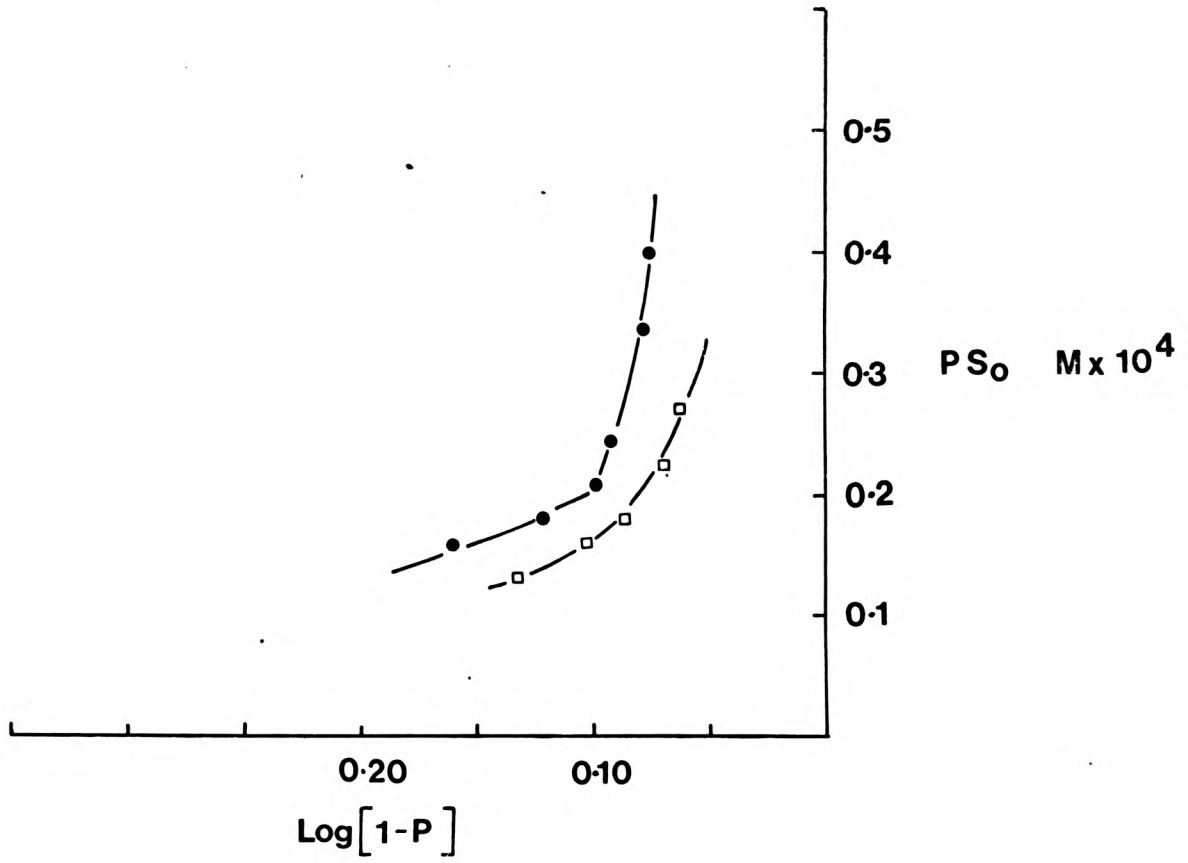
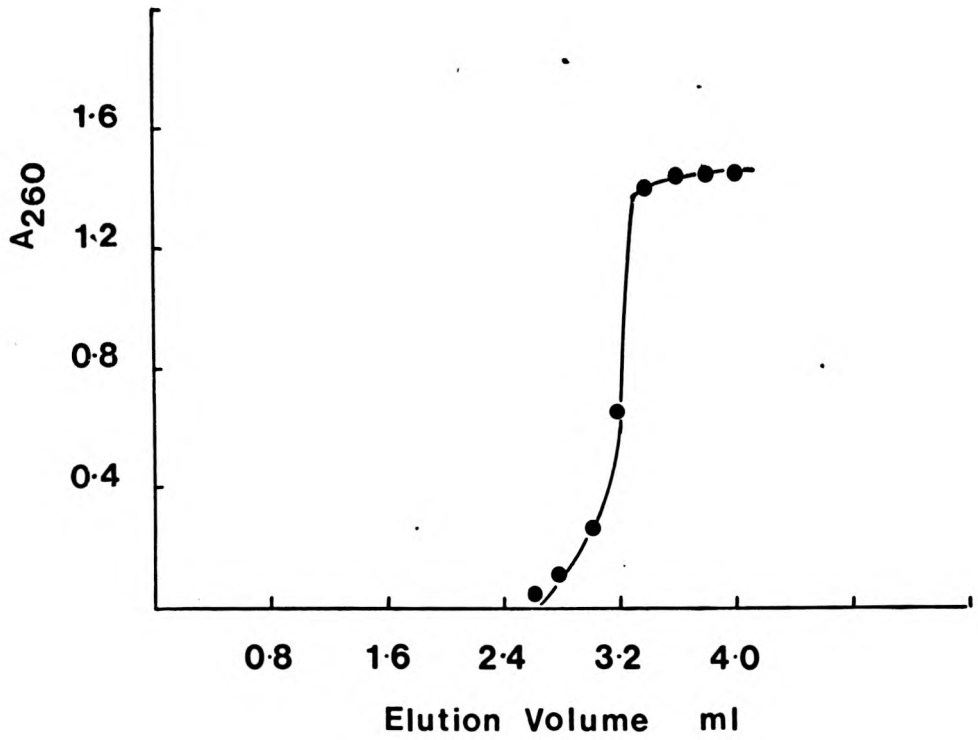

0·5
0·4
0·3
0·2
0·1
PS$_o$ M x 10^4
0·20
0·10
Log[1-P]

FIGURE 29

Demonstration of piston flow of substrate solution through a packed bed (6.8 x 0.9) of polynucleotide phosphorylase immobilized on celite-560. The packed bed was perfused with a solution of ADP and 0.10 ml fractions were collected.


1·6
1·2
0·8
0·4
A260
0·8
1·6
2·4
3·2
4·0
Elution Volume ml

The enzyme is similar to polynucleotide phosphorylase in that it can add monomer to an oligomeric or polymeric primer. The authors presented data on the effect of flow rate on the conversion of substrate by packed beds of the enzyme but they did not plot the data in the form of Equation 2. When that is done, it can be seen that at higher flow rates, the immobilized phosphorylase exhibits similar behaviour to that of polynucleotide phosphorylase (Figure 30). The deviation from linearity is most marked at high flow rates and low substrate concentrations.

Poly (5-chloro cytidylic acid) has recently been prepared (Eaton and Hutchinson, 1972) and complexes of poly (Cl^5C) and Poly(I) are of interest as possible interferon inducers and it was decided to study the polymerization of Cl^5 CDP by a packed bed of immobilized polynucleotide phosphorylase. The results of a typical experiment are shown in Figure 27, where the data is plotted according to Bar-Eli and Katchalski (1963). It can be seen that ADP is polymerized to a greater extent than either CDP or Cl^5 CDP. The polymers were characterized by gel electrophoresis and found to have the following S values, poly (A) 8S, poly(C) 12S and poly(Cl^5C) 6S. These values compare favourably with the values for polymers prepared with the free enzyme.

The performance of the immobilized enzyme was then studied in a batch reactor. The polymerization of three substrates was studied; ADP, CDP and ho^5 CDP. ho^5CDP and poly (ho^5C) have recently been prepared and characterized by Eaton and Hutchinson (1973). ho^5CDP is a poor substrate for polynucleotide phosphorylase, requiring large amounts of enzyme for successful polymerization. The polymerization of ho^5 CDP provides a model system for the polymerization of atypical substrates by immobilized polynucleotide phosphorylase. The polymerization of ADP in a batch reactor is shown in Figure 31.and it can be seen that the extent of polymerization is proportional to the amount of enzyme present. The polymerization of CDP and ho^5 CDP by the immobilized enzyme was studied and it was found that CDP was a much better substrate than ho^5 CDP. In a typical experiment, the yield of poly(C) was 3 x higher than the yield of poly(ho^5C).

FIGURE 30

Kinetic behaviour of glycogen phosphorylase immobilized on porous glass. Data of Marshall and Walter, 1972 plotted according to Hornby, Lilly and Crook, 1966.

—— ⊘ —— ⊘ —— flow rate = 0.50 ml min^{-1}

—— ○ —— ○ —— flow rate = 0.65 ml min^{-1}

—— □ —— □ —— flow rate = 0.90 ml min^{-1}

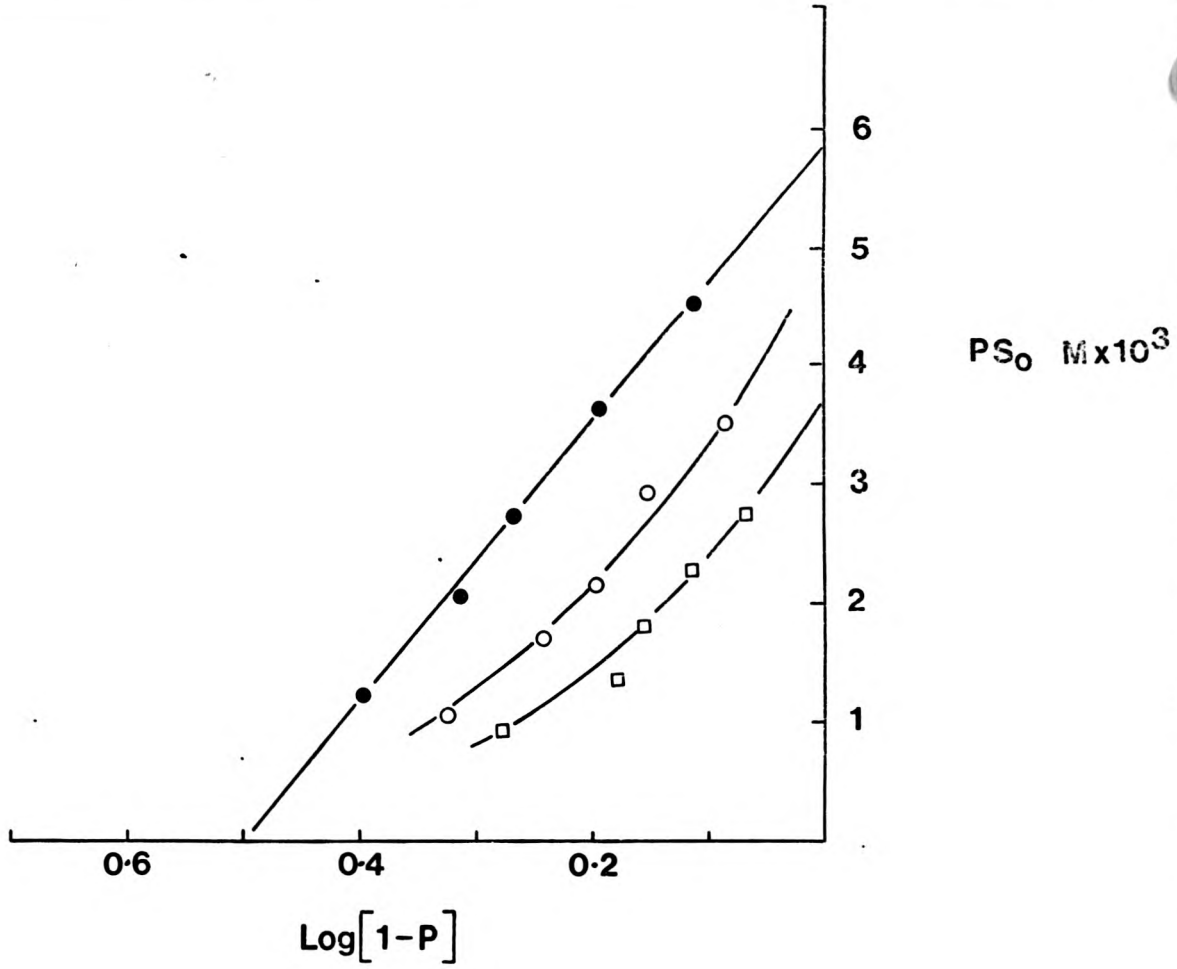

6
5
4
3
2
1
PS$_o$ Mx10^3
0·6
0·4
0·2
Log[1−P]

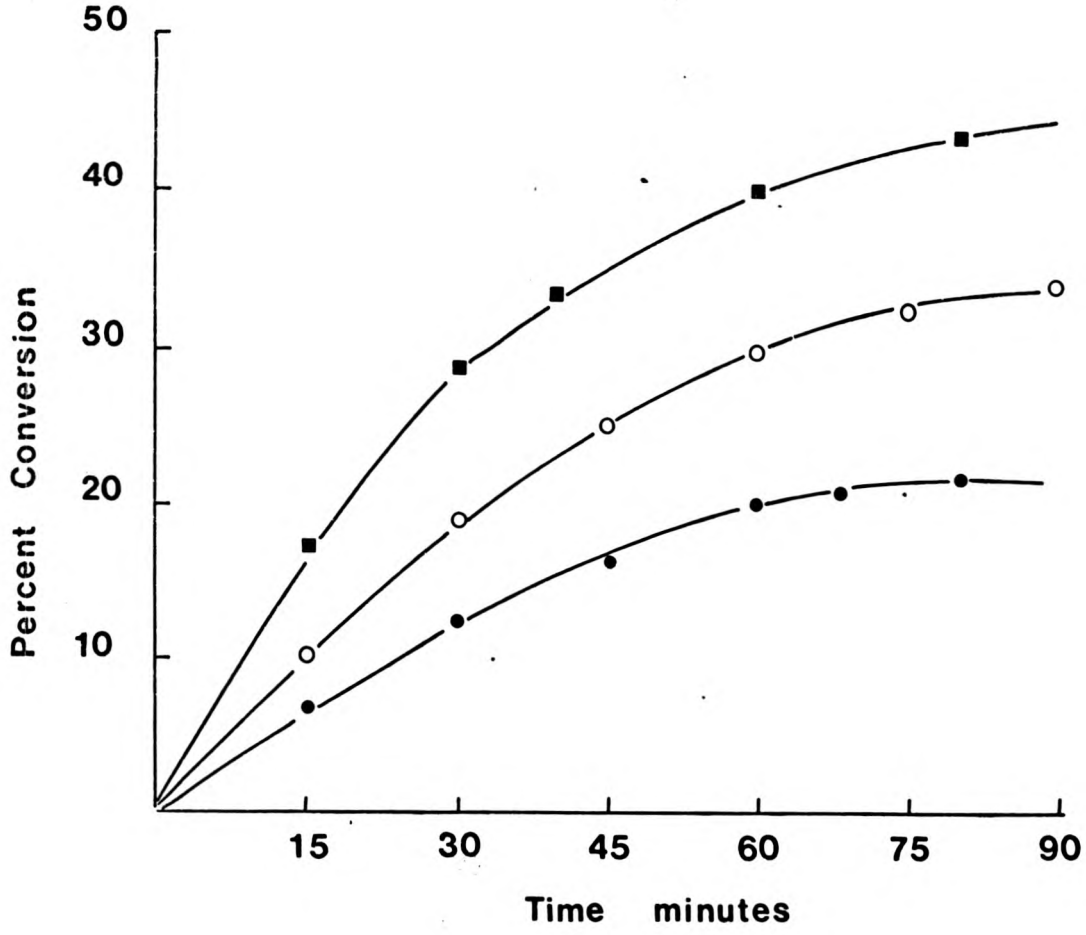
FIGURE 31

Polymerization of ADP by polynucleotide phosphorylase from E. coli immobilized on celite-560. Total volume = 20 ml. Substrate concentration = 2.5×10^{-4} M.

—■—■— Enzyme concentration = 9.75 mg/ml

—O—O— = 6.50 mg/ml

—●—○— = 3.25 mg/ml


50
40
30
20
10
Percent Conversion
15
30
45
60
75
90
Time minutes

Primer dependency of immobilized polynucleotide phosphorylase

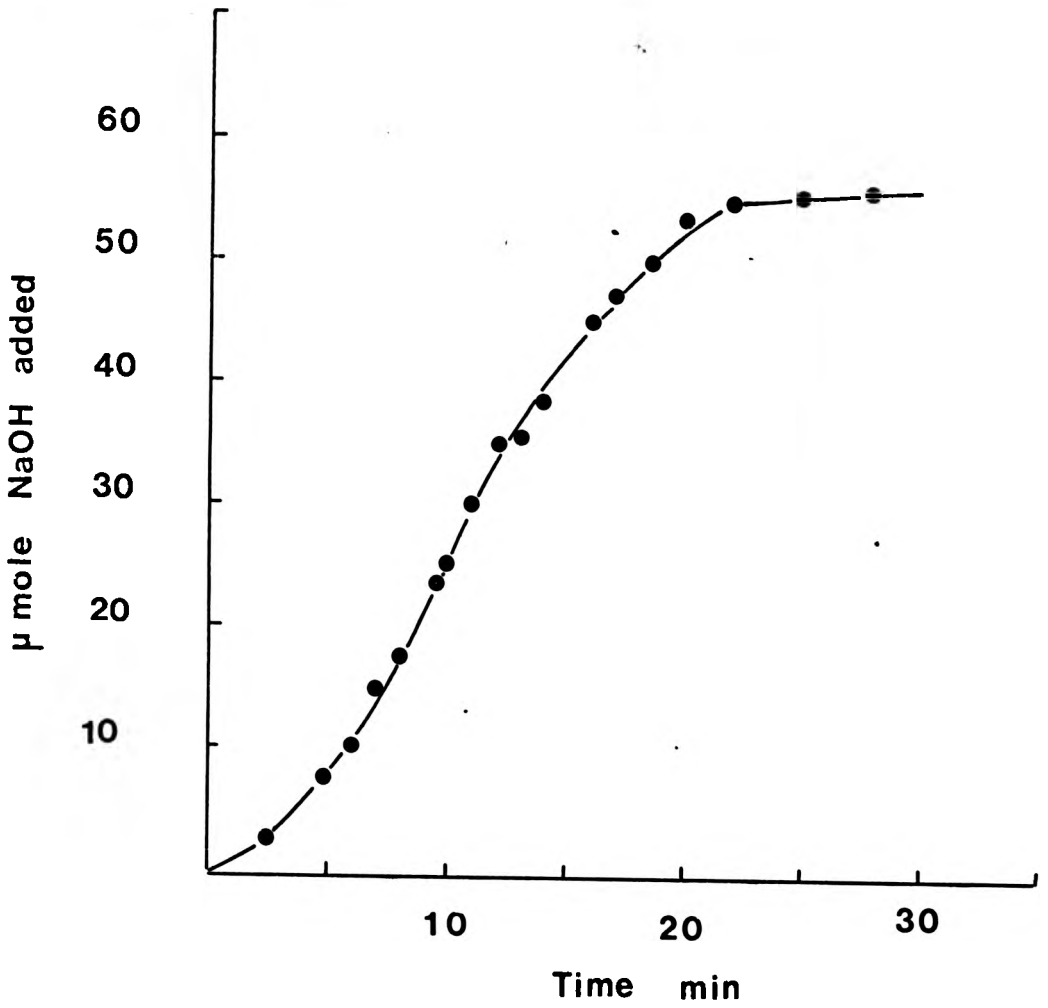
Polynucleotide phosphorylase can be rendered primer dependent by a variety of treatments such as trypsin digestion (Klee and Singer, 1968b). The primer dependent enzyme can be used in the synthesis of defined sequence oligoribonucleotides (Thach and Doty, 1965a). It was therefore of interest to study the effect of trypsin digestion on the activity of the immobilized enzyme.

The reaction of the immobilized enzyme with trypsin was followed by a pH stat method as described in the Methods Section. A typical reaction is shown in Figure 32. Polynucleotide phosphorylase from M. luteus was bound to cyanogen bromide activated Sepharose-4B and the derivative used had a protein content of 54.4 mg/g and an activity of 6.74 units/g. It can be seen that reaction between trypsin and the immobilized enzyme was complete in 25 minutes. After thorough washing, the conjugate was stored in polymerization buffer in the absence of 2-mercaptoethanol and assayed for polymerization activity. The conjugate was then incubated in buffer containing 50 mM 2-mercaptoethanol for 60 minutes and assayed at a final concentration of 10 mM 2-mercaptoethanol. The trypsin treated derivative showed a pronounced lag phase and a lower level of activity. Preincubation of the derivative in 2-mercaptoethanol resulted in the restoration of the polymerization activity. This is in accord with the results obtained by Klee and Singer (1968b).

One of the disadvantages of the immobilized enzyme is the difficulty of handling small reaction volumes. This is a particular disadvantage in the case of primed reactions where high concentrations of primer are required. Many of the literature preparations of defined sequence oligonucleotides are carried out in volumes of less than 100 µl and these volumes cannot be readily handled when the enzyme is immobilized on porous bead structures. One possible method of handling small reaction volumes would be to carry out the reaction in nylon capillary tubes in which the enzyme was covalently bound to the inner surface of the tube.

FIGURE 32

Polynucleotide phosphorylase from M. luteus immobilized on Sepharose 4B – modification by trypsin digestion. Immobilized enzyme (78 mg) was suspended in water (25.0 ml) and the pH was adjusted to 8.0 and the temperature was maintained at 25°. A solution of trypsin in water (300 µl, 0.40 mg/ml) was added and the pH of the suspension was maintained at 8.0 by the addition of 0.10 M sodium hydroxide. When there was no further change in pH, the immobilized enzyme was washed on a filter to remove traces of trypsin and stored in polymerization buffer.


60
50
40
30
20
10
μ mole NaOH added
10
20
30
Time min

Attempts to prepare an immobilized derivative of B. stearothermophilus polynucleotide phosphorylase

Several attempts were made to obtain stable and active derivatives. The enzyme was covalently linked to cyanogen bromide activated Sepharose and to aminoalkyl glass through the bifunctional reagent, glutaraldehyde, using the procedures described previously. Attempts were also made to attach the enzyme to nylon powder which had been activated with dimethyl sulphate (Hornby et al., 1974; Sundaram, 1974). In all three cases, protein was covalently linked to the matrix but the conjugates were inactive.

The results suggested that the activated matrix was reacting with an amino acid residue which was essential for the activity of the enzyme. Cyanogen bromide activated polysaccharides have been shown to react predominantly with lysine residues (Axen, Porath and Ernback, 1967). Similarily, glutaraldehyde reacts with lysine residues (Habeeb, 1967) and dimethyl sulphate activated nylon reacts with nucleophiles such as the ε-amino group of lysine (Sundaram, 1974). It is possible that the immobilization techniques used resulted in reaction with a lysine group which was essential for the activity of the enzyme. It was decided to study the effect of a lysine modifying reagent on the activity of the enzyme in free solution. The reagent chosen was trinitro-benzenesulphonic acid which has been reported as modifying lysine residues (Freedman and Radda, 1968). A sample of the enzyme was dialysed against 0·20 M sodium bicarbonate pH 8.5 and was then treated with an excess of reagent. The reaction was followed by observing the change in absorbance at 340 nm and aliquots were removed and assayed for polymerization activity. It was found that the enzyme rapidly lost activity, losing 20% of the original activity in 10 minutes. It was concluded that a lysine residue was essential for the activity of the enzyme and that this lysine residue reacts with the reactive group on the activated matrix.

The use of large oligonucleotides as primers for polynucleotide phosphorylase from B. stearothermophilus

The use of polynucleotide phosphorylase from M. luteus in the synthesis of short oligonucleotides of defined sequence has been described (Thach and Doty, 1965 a,b) Syntheses involving the use of larger oligonucleotide primers have been hampered by the problem of base-pairing between primer and the newly formed polymer. A possible method of overcoming this problem would be to carry out the reaction at temperatures higher than the melting temperature of the primer-polymer complex. There has recently been much interest in the enzymology of the thermophilic bacteria (Singleton and Amelunxen, 1973) and polynucleotide phosphorylase from B. stearothermophilus has been isolated and characterized (Hutchinson and Wood, 1973).

Oligonucleotides (Chain length 15) were used as primers for polynucleotide phosphorylase from B. stearothermophilus. It was hoped to synthesize analogues of the poly(C) tract which was recently discovered in the genome of Encephalomyocarditis virus (Porter, Carey and Fellner, 1974). Attempts were also made to use t-RNA as a primer for the enzyme following the observation that Mengo virus RNA could be charged with histidine (Salomon and Littauer, 1974). If other viral RNA molecules contained a t-RNA like structure at their 3'-terminal, it would be of interest to prepare t-RNA analogues as possible anti-viral agents.

The enzyme was isolated by affinity chromatography and preliminary studies were carried out in order to establish the optimum conditions for polymerization. The pH profiles for the polymerization and phosphorolysis reactions are similar to those obtained with polynucleotide phosphorylase from other sources. Similar pH profiles for the polymerization of ADP were obtained in the presence of the oligonucleotide primer $(Ap)_4A$. The substrate specificty of the enzyme was then studied and the results are shown in Table 8. The enzyme exhibits a lower activity with pyrimidine substrates than with purine substrates. ADP is the most efficient substrate for the polymerization reaction but CDP is only polymerized in the presence of a suitable primer. A similar reduction in the ability to polymerize UDP and CDP was observed when M. luteus polynucleotide phosphorylase was treated

TABLE 8

Substrate Specificity of polynucleotide phosphorylase from *B. Stearothermophilus*

Phosphorolysis	μmole/hour
Poly (A)	36.50
Poly (I)	5.00
Poly (C)	0.1
Poly (U)	0.1
Poly (G)	0.1

Polymerization

	- primer(μmole/hour)	+ primer (Ap) A	Enhancement
ADP	1.32	1.68	1.27
IDP	1.22	1.41	1.16
GDP	0.035	0.216	6.17
CDP	0.0024	0.720	300
UDP	0.084	0.440	5.23

with trypsin (Fitt and Fitt, 1967). The presence of endogenous proteolytic enzymes can also lead to an increase in primer dependency (Klee and Singer, 1967). It was therefore of interest to examine the crude and purified enzymes for proteolytic activity.

There was no detectable esterase, amidase or peptidase activity in the purified enzyme. Two crude extracts were prepared, one in which the cells were broken by lysozyme and deoxyribonuclease treatment and one in which the cells were broken by osmotic shock. There was no esterase or peptidase activity present in either of the extracts. However, the lysozyme-deoxyribonuclease treated extract did show activity against BAPNA indicating the presence of an amidase (0.003 u/ml, 1 unit = 1μmole/min). Amidase activity was not present in the extract prepared by osmotic shock. The level of proteolytic activity in the B. stearothermophilus extracts is much lower than that observed in E. coli (Pacaud and Uriel, 1971; Regnier and Thang, 1972) and would indicate that the properties of the purified enzyme are those of the native enzyme.

The enzyme is not base specific with respect to primer as $(Ip)_5I$, $(Up)_5U$ and $(Ap)_5A$ all function as equally good primers for the polymerization of CDP. $d(pT)_{11}T$ has also been used as a primer for the polymerization of CDP. These studies of substrate specificity indicated that the enzyme would be suitable for introducing oligo(C) at the 3'terminal of oligonucleotide primers. In addition, the specificity for poly(A) in the phosphorolysis reaction could make the enzyme useful in the study of the poly (A) region of m-RNA.

Use of dihydroxyboryl Sepharose in the isolation of long chain oligonucleotide primers

Dihydroxyboryl derivatives of aminoethyl cellulose and carboxymethyl cellulose have been prepared and used for the isolation of ribonucleotides with intact 3' hydroxyl groups (Wieth, Wiebers and Gilham, 1970; Rosenberg, 1974). Ion exchange celluloses are not the most suitable materials for this purpose since if there is incomplete substitution of the matrix, the remaining charged groups will interfere with the purification procedure. It was therefore decided to attempt to bind m-aminophenylboronic

acid to cyanogen bromide activated Sepharose-4B. The optimum conditions for coupling were established by carrying out small scale reactions over a range of pH values. The extent of binding was determined by measurement of the absorbance of the washings at 293 nm. The optimum pH for binding was found to be pH 6.0 (Figure 33). Amines are normally bound to cyanogen bromide activated Sepharose in the pH range 8.0 - 9.0 but it has recently been shown that the optimum pH for coupling is related to the pKa of the amine in the following way (Kagedal, 1975).

Optimum pH for binding = pKa + 1.5 pH units.

The literature values of pK for m-aminophenylboronic acid are 4.46 and 8.12 (Clear and Branch, 1938) and the observed pH optimum is therefore in good agreement with the relationship proposed by Kagedal. The conjugate prepared by this method contained 12.05 m mole of ligand/g matrix compared with 0.62 m mole/g obtained when aminoethyl cellulose was used as the matrix.

Model studies were carried out with oligonucleotides of the deoxy and ribo series. The results are shown in Figure 34 and it can be seen that it is possible to separate oligonucleotides on the basis of the presence or absence of 2', 3' diol groups. The presence of an unesterified 3' hydroxyl group is essential if the oligonucleotide is to function as a primer for polymerization (Godefroy, Cohn and Grunberg-Manago, 1970) and the use of dihydroxylboryl Sepharose ensures that the primer is free of material containing 3' phosphates which could cause inhibition.

Incorporation of CDP into oligo(I) primers by polynucleotide phosphorylase from B. stearothermophilus

Oligo(I) was prepared as described in the Methods Section and was found to have a chain length corresponding to 20 residues. The utilization of this primer was compared with that of a shorter oligonucleotide primer, $(Ip)_5I$. It was found that the shorter primer was more effective and that the initial rate in the presence of $(Ip)_5I$ was four times the rate in the presence of the larger oligonucleotide.

The products were isolated and the chain length of the incorporated oligo(C) region was determined by pancreatic ribonuclease

FIGURE 33

Coupling of m-aminophenyl boronic acid to cyanogen bromide activated Sepharose - 4B.

Aliquots of Sepharose-4B (3.3 ml) were activated with cyanogen bromide (1g) and added to solutions (20 ml) containing the ligand. Acetate buffers were used in the pH range 3.0 - 5.5, phosphate buffers were used in the pH range 5.5 - 8.0 and borate buffers in the range 8.0 - 9.5. The suspensions were stirred at 4^{o} for eighteen hours. Unbound ligand was removed by filtration and washing with water (250 ml). The extent of binding was determined from measurements of the absorbance of the washings at 293 nm.

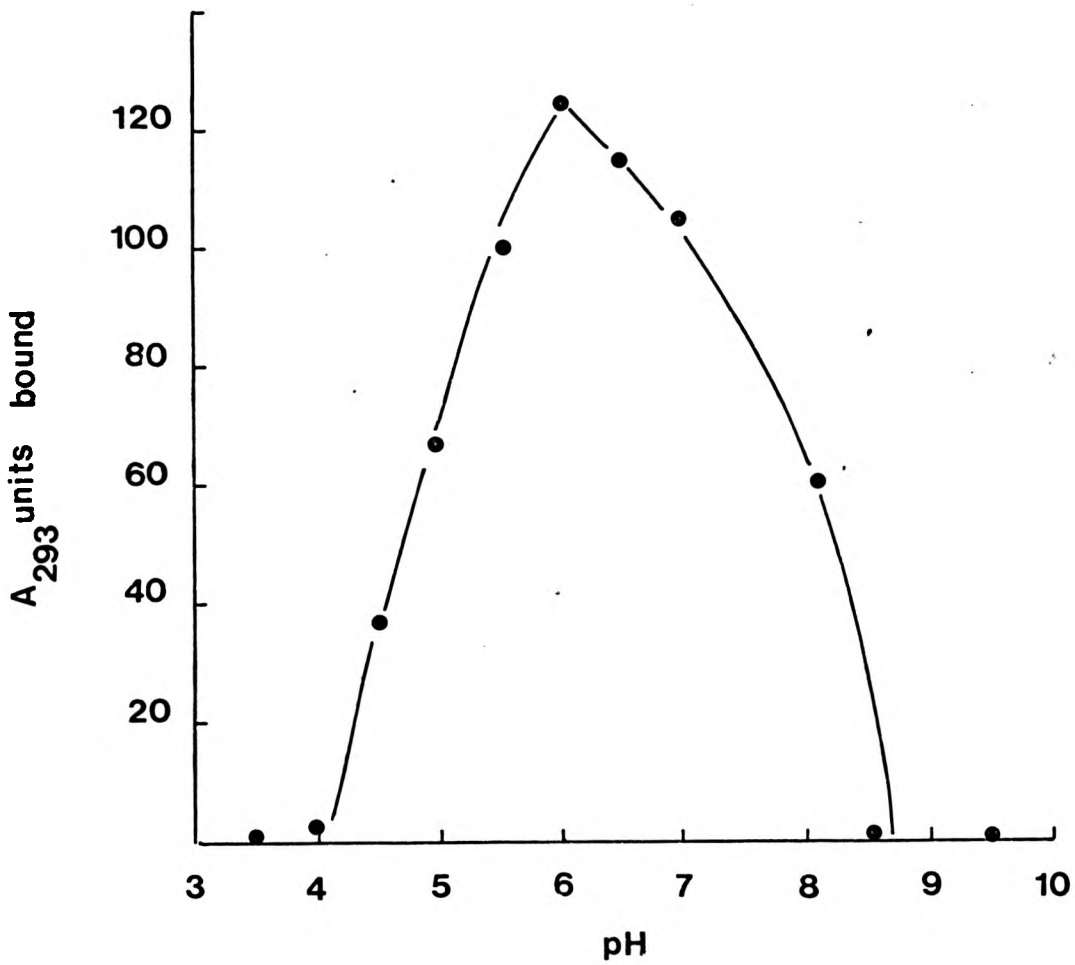

A_{293} units bound
120
100
80
60
40
20
3
4
5
6
7
8
9
10
pH

FIGURE 34

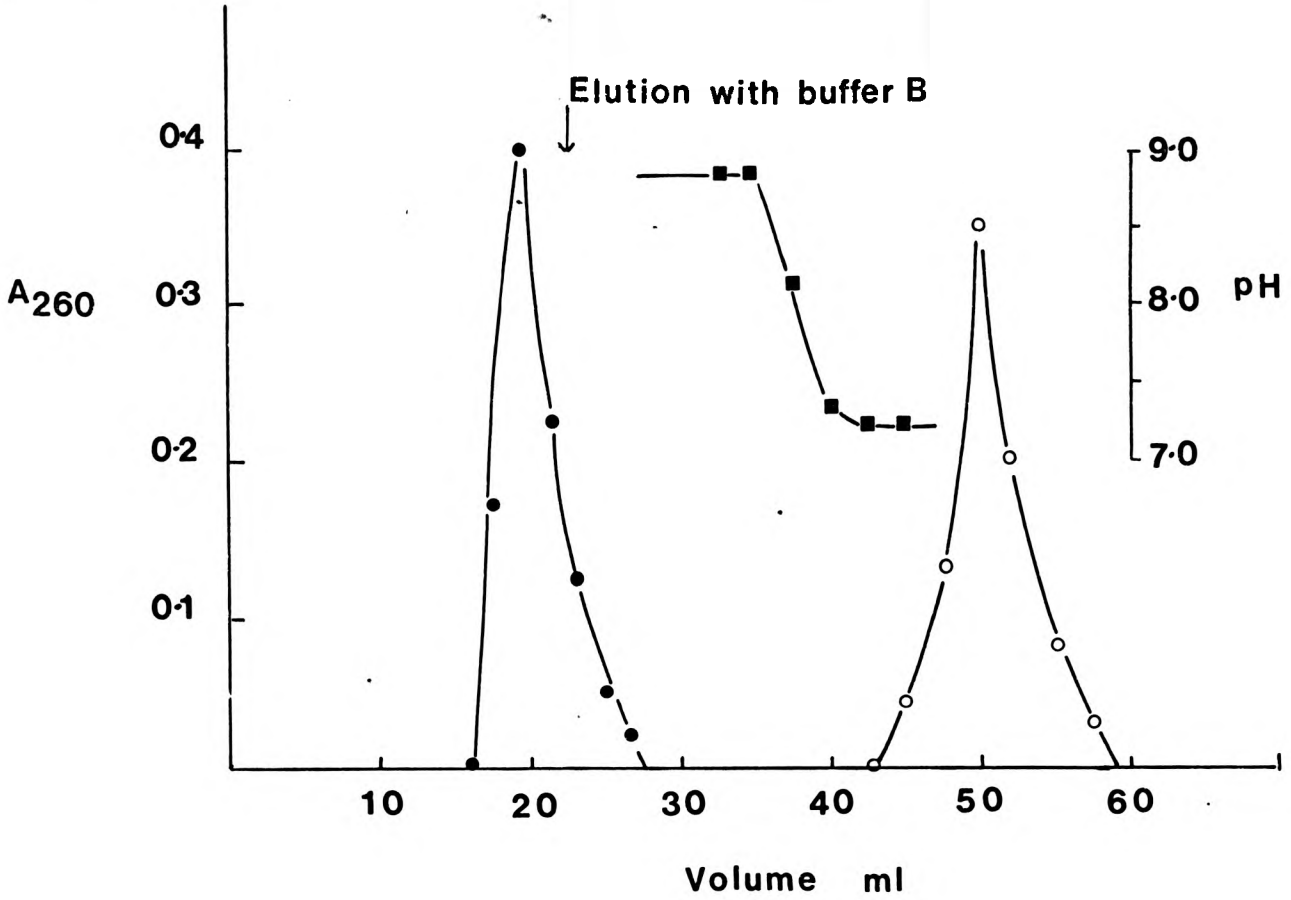
Separation of oligo (dC) from oligo (rA) on a column of dihydroxyboryl Sepharose.

A column of dihydroxyboryl Sepharose (0.9 x 40 cm) was equilibrated with Buffer A. A mixture of oligo (dC) (10 A260 units) and oligo (rA) (10 A260 units) in Buffer A (100 µl) was applied to the column and eluted with Buffer A. Elution with Buffer B was commenced after unbound material had been eluted.

—— o —— o —— oligo (dC)

—— o —— o —— oligo (rA)

—— ▫ —— ▫ —— pH


Elution with buffer B
A_{260}
0·4
0·3
0·2
0·1
9·0
8·0
7·0
pH
10
20
30
40
50
60
Volume ml

digestion and separation of the products by electrophoresis. It was found that 40 residues were incorporated into the $(Ip)_5I$ primer while only 4 residues were incorporated into the longer primer. The products were further characterized by changes in their UV spectra (Table 9).

The incorporation of cytidine residues into oligonucleotides with chain length greater than ten residues was disappointingly low. The use of primer dependent polynucleotide phosphorylase in synthetic work is therefore limited to syntheses starting with short chain length oligonucleotides. However, the ability to incorporate a small number of residues onto the 3' terminal of a polynucleotide could be of use as a sequencing technique. Radioactively labelled residues could be incorporated into the 3'-terminal of an unlabelled polynucleotide. After partial digestion with ribonucleases, oligonucleotides containing nucleotide sequences near the 3'-terminal could be obtained and identified by standard techniques.

The use of t-RNA as a primer for polynucleotide phosphorylase from B. stearothermophilus

E. coli t-RNA was purified by chromatography on dihydroxy-boryl Sepharose. Low molecular weight impurities were first removed by gel filtration on Sephadex G-200. The material contained a number of amino acid acceptor activities, of which leucine and serine were the most abundant. Purified phenylalanine specific t-RNA from brewer's yeast was used in studies which involved sequencing of the product.

The effect of t-RNA on the polymerization is shown in Figure 35. It can be seen that t-RNA is effective as a primer. Preliminary studies on priming by t-RNA were carried out in the presence of magnesium ions. In an effort to increase the rate of incorporation of substrate, the effect of manganese ions was studied. In the presence of manganese ions the rate of incorporation of substrate was double the rate observed with magnesium ions. The optimum manganese concentration was found to be 5 mM and maganese was used in all the following experiments.

The effect of varying the concentration of t-RNA was then studied. The ratio $\frac{\text{Primer}}{\text{Enzyme}}$ appears to be critical (Figure 36A). The maximum rate of polymerization is obtained when the primer is in a

TABLE 9

Spectroscopic properties of oligo(I) - oligo(C) hybrids

	A250/A280	λmax
$(Ip)_5I$	5.82	248
I_6C_{40}	0.975	274
$I_6C_{40}I_n$	2.85	254

0.05M sodium cacodylate, 0.10M sodium chloride pH 7.0

FIGURE 35

t-RNA as a primer for the polymerization of CDP by polynucleotide phosphorylase from B. Stearothermophilus

Each reaction (100 µl) contained [14C]-CDP (0.6 µmole, 0.025 µCi) and enzyme (40 µg, 54 u) in 0.10 M glycine, pH 9.2 containing 10 mM magnesium chloride and 2 mM mercaptoethanol. Aliquots (5 µl) were removed for analysis by TLC. t = 65°.

—○—○— + t-RNA (1.0×10^{-9} mole)

—□—■— - t-RNA

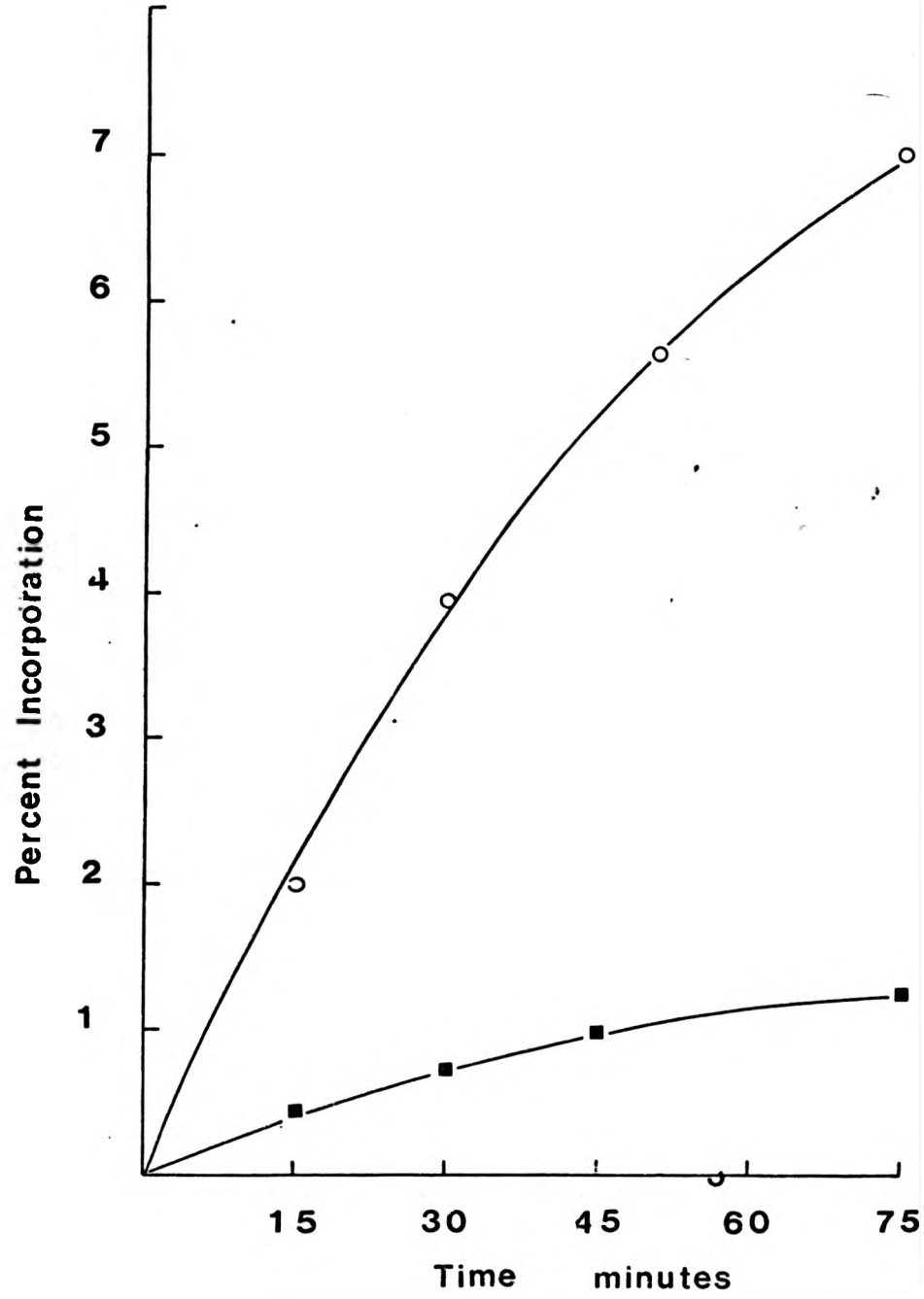

Percent Incorporation
7
6
5
4
3
2
1
15
30
45
60
75
Time minutes

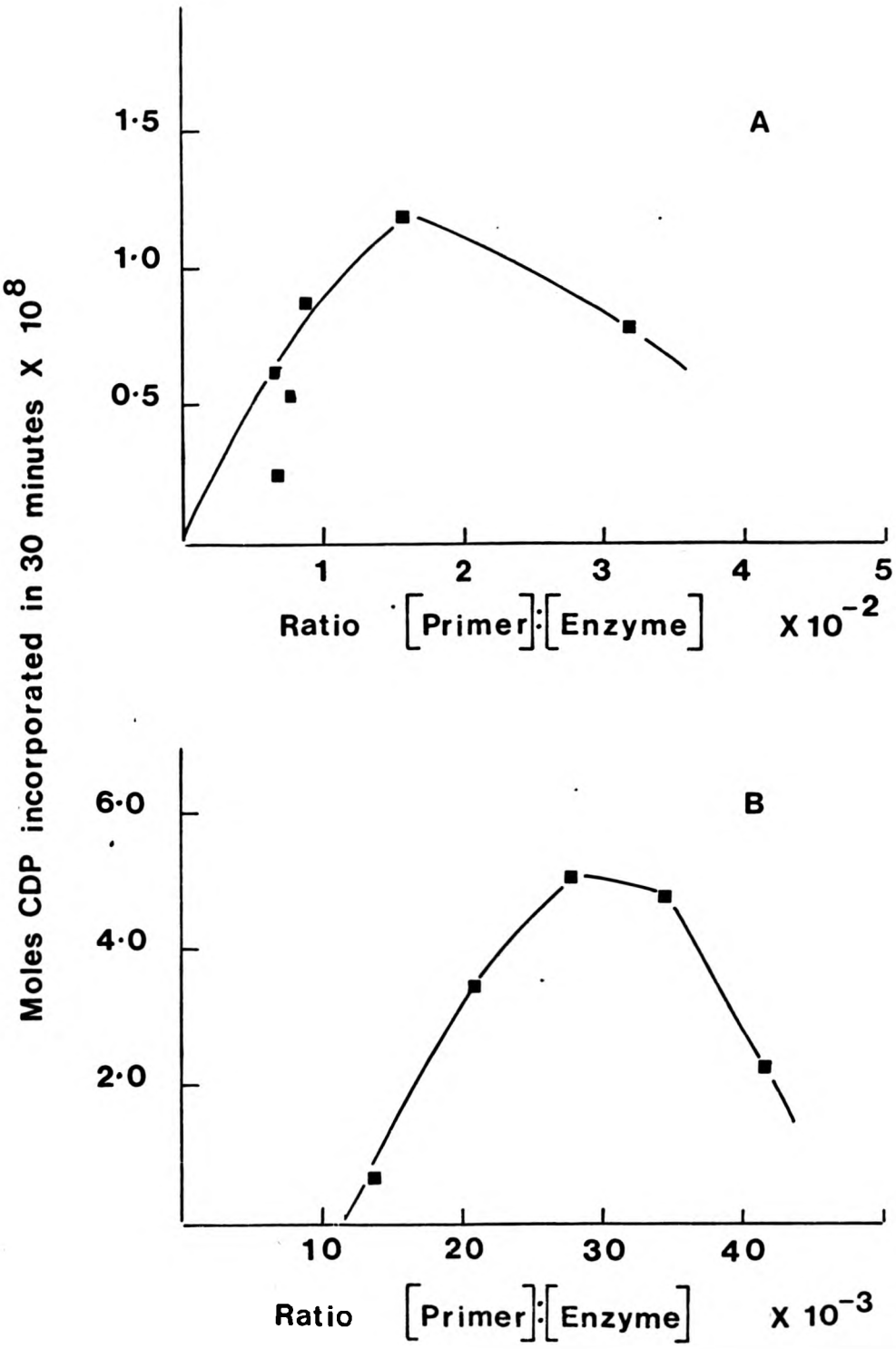
FIGURE 36

Incorporation of CDP into t-RNA and $(Up)_5U$.

Each reaction (100 µl) contained CDP (0.6 µmole) in 0.10 M glycine, pH 9.2 containing 5 mM manganous chloride, 2mM mercaptoethanol and 1 mM EDTA and various amounts of enzyme and primer, t = 65°

A primer = t-RNA

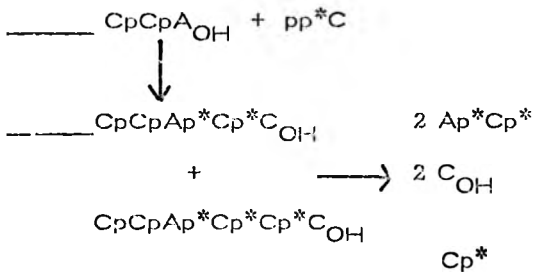
B primer = $(Up)_5U$


A
1·5
1·0
0·5
1
2
3
4
5
Ratio [Primer]:[Enzyme] X 10⁻²
B
6·0
4·0
2·0
10
20
30
40
Ratio [Primer]:[Enzyme] X 10⁻³
Moles CDP incorporated in 30 minutes X 10⁸

hundredfold excess over enzyme. At higher and lower levels of primer, the rate of polymerization decreases. A similar result is obtained when $(Up)_5U$ is used as a primer (Figure 36B), but in this case, the optimum ratio of primer to enzyme is two orders of magnitude greater. Similar effects were observed when manganese ions were replaced by magnesium ions.

Preliminary studies indicated that up to five cytidine residues could be incorporated into t-RNA. In order to study the products in detail, it was decided to prepare $\left[^{32}P\right]$-CDP and to incorporate it into phenylalanine specific t-RNA. The product could then be analysed using standard sequencing techniques (Sanger, Brownlee and Barrell, 1965). A t-RNA-oligo(C) hybrid was prepared and isolated by gel filtration on Sephadex G-50. 2% of the total counts eluted at the void volume. This material was lyophilized and then digested with pancreatic ribonuclease. Two products were obtained on electrophoresis, one corresponding to ApCp and one corresponding to Cp. The products were identified using markers and by comparison of their mobilities with the mobility of the xylene cyanol marker. The amount of material present was determined by scintillation counting and the ratio ApCp : Cp was found to be 4 : 1. The ApCp spot was further characterized by alkaline hydrolysis which gave two products corresponding to Ap and Cp. The ratio Ap : Cp was 1.2 : 1.

The sequence of phenylalanine specific t-RNA has been determined (Raj Bhandary et al., 1967) and the sequence at the 3'terminal is $-CpCpA_{OH}$. If the t-RNA functions as a primer for polynucleotide phosphorylase, the expected product would be $-CpCpAp^*Cp^*Cp^*\ldots C_{OH}$ where the asterisk indicates the presence of the $\left[^{32}P\right]$ label. Pancreatic ribonuclease digestion should yield Ap^*Cp^* and Cp^*. The observed ratio of the products suggests that only a small number of residues is being incorporated into the primer. The observed 4 : 1 ratio could be interpreted in the following way:



These results indicate that the length of the attached oligo(C) is at most three or four residues long. This is shorter than the value obtained using $[^{14}C]$-labelled substrate and heterogenous t-RNA as primer. The lower value obtained using $[^{32}P]$-labelled substrates could have been due to the presence of impurities formed during the conversion of the tripnosphate to the diphosphate. It would appear that while polynucleotide phosphorylase from B. stearothermophilus can utilize t-RNA and other long oligonucleotides as primers, the number of residues incorporated is small. As discussed in the section on oligo(I) primers, labelling of RNA molecules with CDP could provide a means of identifying the 3' terminal of the molecule. Polynucleotide phosphorylase from M. luteus has been used to label the products of T_1 ribonuclease digestion (Szeto and Soll, 1974). Unlabelled t-RNA was digested with T_1 ribonuclease and with alkaline phosphatase and the oligonucleotides were then utilized as primers for the incorporation of $[^{32}P]$- labelled CDP by primer dependent polynucleotide phosphorylase. The labelled oligonucleotides were again digested with T_1 ribonuclease and purified by two dimensional paper electrophoresis. The longest oligonucleotides labelled by this method were eleven residues long and the use of B. stearothermophilus polynucleotide phosphorylase allows the labelling of intact RNA molecules. Polynucleotide phosphorylase has also been used to identify the 3' terminal by stepwise phosphosphorolysis of the terminal residues but the method could not be applied to oligonucleotides longer than twelve residues (Kaufmann, Grosfeld and Littauer, 1973).

Appendix

The purification of an endonuclease from pig liver nuclei and its use in the preparation of oligonucleotide primers

The most convenient method of preparing oligonucleotide primers is the degradation of polynucleotides which can be achieved by alkaline or enzymatic hydrolysis. Complete alkaline hydrolysis will result in the formation of mononucleotides with a 3' phosphate group but conditions can be selected which result in oligonucleotide products. These phosphorylated oligomers will not function as primers for polynucleotide phosphorylase until the terminal phosphate group has been removed with alkaline phosphatase. The oligonucleotides can be fractionated by chromatography on DEAE-cellulose.

Partial digestion of polynucleotides by endonucleases can also be used to prepare oligonucleotides. Of the enzymes commonly used, only the endonuclease from pig liver forms oligonucleotides which can be used as primers for polynucleotide phosphorylase without further alkaline phosphatase treatment. The enzyme was first described by Heppel (1966) and since then many workers have used crude preparations of the enzyme to prepare oligonucleotide primers (Mackey and Gilham, 1971; Bennett *et al.*, 1973). The enzyme is normally used to prepare primers with a chain length of less than five residues but in the present study the enzyme was used to prepare oligonucleotides with chain lengths of 15 - 25 residues

Materials and Methods

Reagents

Alkaline phosphatase (EC 3.1.3.1.) from *E. coli* (BAPF grade) was obtained from Worthington Biochemical Corporation. Other materials have been described in the previous section.

Enzyme purification

The enzyme was isolated from pig liver nuclei essentially as described by Heppel (1966). This gave a preparation with an activity of 2 units/mg (1 unit = 1 μmole of adenylic acid liberated/hour) and a recovery of 25% of the original activity. The enzyme was then further purified by gel filtration on Sephadex G-200. A column of Sephadex G-200

(60 x 2.5 cm) was equilibrated with 0.05 M MOPS pH 7.0 containing 1 mM 2-mercaptoethanol. Enzyme (5 ml) was applied to the column and eluted with the same buffer. Fractions containing activity were pooled and stored at -20°.

Assay of nuclease activity

Initially, the optical assay of Heppel (1966) was used. The assay mixture contained in 200 µl : 0.05 M MOPS, pH 7.2, 2 mM magnesium chloride, 1 mM 2-mercaptoethanol and poly (A) (4 mg/ml). Enzyme (50 µl) was added and the mixture incubated at 37° for sixty minutes. An aliquot (50 µl) was added to cold 3% perchloric acid (0.45 ml). After ten minutes at 4°, the precipitate was removed by centrifugation. A sample (0.20 ml) was taken from the supernatant and made up to 2 ml with water and the absorbance was measured at 257 nm. In later studies, a modification using radioactive polynucleotides was used as it was found that this method gave a more reproducible value.

[^{14}C]— labelled polynucleotides were prepared from the nucleoside diphosphate using polynucleotide p[illegible] Hutchinson, 1972). A typical assay contained [^{14}C]— poly(A) (50 l, 4 mg/ml, specific activity 1.46 x 10^6 dpm/µ mole) in 0.05 M MOPS pH 7.2 containing 2 mM magnesium chloride and 1 mM 2-mercaptoethanol. Enzyme (20 µl) was added and the mixture was incubated at 37° for sixty minutes. Aliquots (10 µl) were applied to glass fibre discs (Whatman GF/C) and the nucleic acid was precipitated by the method of Bollum (1966). The radioactivity remaining on the discs was determined in a toluene based scintillation liquid.

A disadvantage of both assays is that they measure the production of small acid soluble oligonucleotides (< 10 residues). It can be shown that the initial products of the reaction are oligonucleotides with a chain length of approximately 20 residues. The production of acid soluble oligonucleotides is not linear with time and this complicates the comparison of fractions during the purification of the enzyme.

Similar assay conditions were used in the study of the action of the enzyme on other polynucleotides. The magnesium ion requirements and pH optima were determined using the assay conditions described except

that the magnesium concentration or pH was varied. Protein was measured by the method of Lowry et al (1951).

Polyacrylamide gel electrophoresis of oligonucleotide primers

7.5% polyacrylamide gels were run in 0.10 M tris-borate pH 8.3 containing 2.5 mM EDTA and 7 M urea. Electrophoresis was carried out at 4° at 100 V and 5 mA/gel using bromophenol blue as a marker dye. The large oligonucleotides isolated from the EMC genome (Porter, Carey and Fellner, 1974) were used to calibrate the gel products.

Isolation of oligonucleotides

Oligonucleotides with chain length of less than ten residues were isolated by chromatography on DEAE cellulose in the presence of 7 M urea (Bennett et al., 1973). Longer oligonucleotides were isolated by gel filtration on Sephadex G-200. Fractions corresponding to various size classes were pooled and characterized by phosphate analysis.

Preparation and assay of a nuclease-Sepharose conjugate

Sepharose-6B was activated with cyanogen bromide as described previously (Axen and Ernback, 1971). Activated Sepharose (20 ml) was added to a solution of the purified enzyme (20 ml, 30 mg protein) in 0.10 M MOPS, pH 7.5. The suspension was stirred at 4° for eighteen hours and was then washed with 1.0 M sodium chloride (500 ml) and water (500 ml). The conjugate was stored at 4° in 0.05 M MOPS, pH 7.2 containing 5 mM 2-mercaptoethanol. The immobilized enzyme was assayed by recirculating substrate solution through a packed bed of the immobilized enzyme (Ford et al., 1972). The reaction was followed by observing the rise in absorbance at the λ max of the polynucleotide using 1 mm path length flow cells.

Results

Purification of the enzyme

The purification of the enzyme is outlined in Table 10 and Figure 37. The most active fraction had a specific activity of 21 units/mg, representing an overall 40 fold purification. Values for the intermediate steps in the purification have not been included as exonuclease activity is also present in the early stages of the purification.

Rechromatography of the most active fractions on Sephadex G-200 and comparison of the elution volume with those of protein standards established the molecular weight of the enzyme as 68,000.

Properties of the purified enzyme

The optimal reaction conditions regarding magnesium concentration and pH are shown in Figures 38 and 39. The optimum magnesium concentration occurs in the presence of one equivalent of Mg^{2+} per phosphate residue in the polynucleotide. The purine homopolymers tended to precipitate at this magnesium concentration and hydrolysis of these polymers was carried out at lower magnesium concentrations.

Poly(A), poly(I) and poly(C) were hydrolysed at similar rates by the purified enzyme. Poly(G) was hydrolysed at a much lower rate. Calf thymus DNA and single stranded polydeoxynucleotides, such as poly(dC), were not hydrolysed. This was determined by the absence of acid soluble products after treatment with the enzyme and by the unchanged profile on Sephadex G-200 gel filtration. Mononucleoside cyclic phosphates, such as adenosine 2'3' cyclic monophosphate and adenosine 3'5' cyclic monophosphate, were not hydrolysed by either the crude or purified enzyme, indicating that the enzymic reaction did not proceed through a cyclic intermediate (Barnard, 1969).

The initial products of the reaction are typical of an endonucleolytic cleavage. A polynucleotide approximately 1000 residues long yields oligonucleotides with chain lengths of 15-25 residues after two hours incubation (Figure 40).

The enzyme is competitively inhibited by oligodeoxynucleotides, such as oligodeoxythymidylic acid. The hydrolysis of poly(A) by the enzyme

TABLE 10

Purification of a ribonuclease from pig liver nuclei

	Protein (mg/ml)	Specific Activity (u /mg)	Yield %
Crude Extract	15.0	0.50	100
Ammonium Sulphate (0-33%)	10.0	2.0	25
Sephadex G-200			
Peak	1.5	21	
pooled	1.5	11	21

FIGURE 37

Purification of pig liver nuclease on Sephadex G-200.

A column (60 x 2.5 cm) of Sephadex G-200 was equilibrated with 0.05 M MOPS, pH 7.2 containing 1 mM 2-mercaptoethanol. Enzyme was applied to the column and eluted with the same buffer. Fractions (2.4 ml) were collected and assayed for protein and enzymic activity.

—— ○ —— ○ —— Protein (mg/ml)

—— □ —— ■ —— Activity (units/ml)

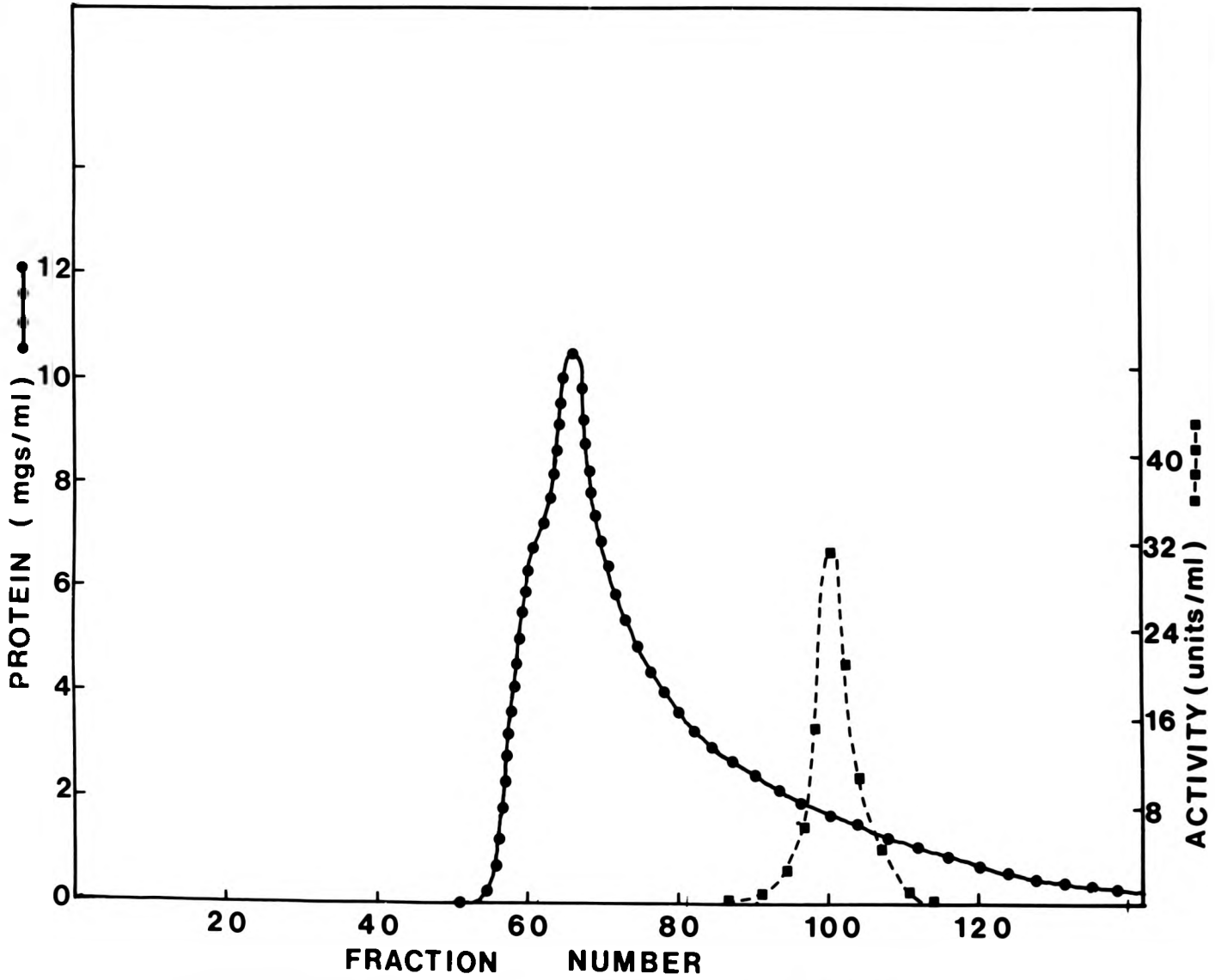

PROTEIN (mgs/ml)
0
2
4
6
8
10
12
ACTIVITY (units/ml)
8
16
24
32
40
20
40
60
80
100
120
FRACTION NUMBER

FIGURE 38

Effect of Magnesium ion concentration on the hydrolysis of poly(C). Incubations(50 µl) were carried out with variable Magnesium (II) concentrations as described in the Methods Section. After one hour, the reactions were stopped by the addition of cold 3% perchloric acid and filtered onto glass fibre discs which after washing were counted in a toluene based scintillation fluid.

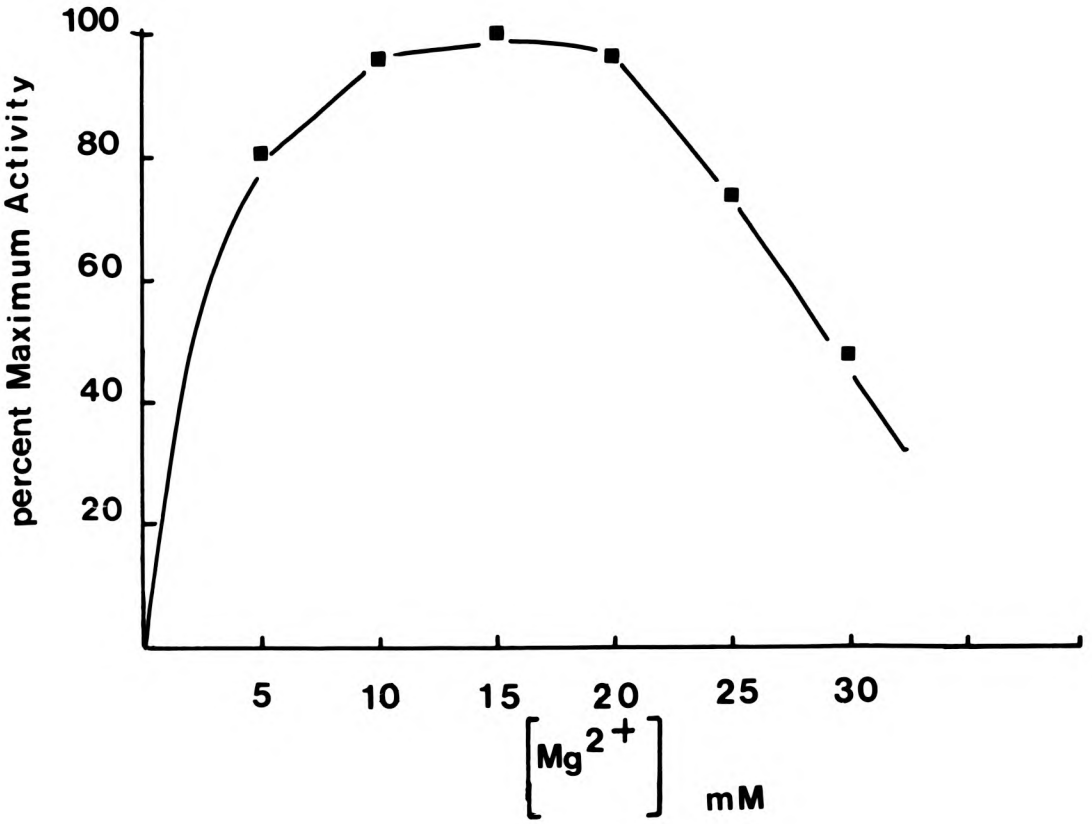

percent Maximum Activity
100
80
60
40
20
5
10
15
20
25
30
[Mg2+]
mM

FIGURE 39

The pH optimum of poly(C) hydrolysis. 50 µl assays were carried out in 0.05M MOPS containing 15 mM magnesium chloride and 15 mM poly(C) at various pH values.

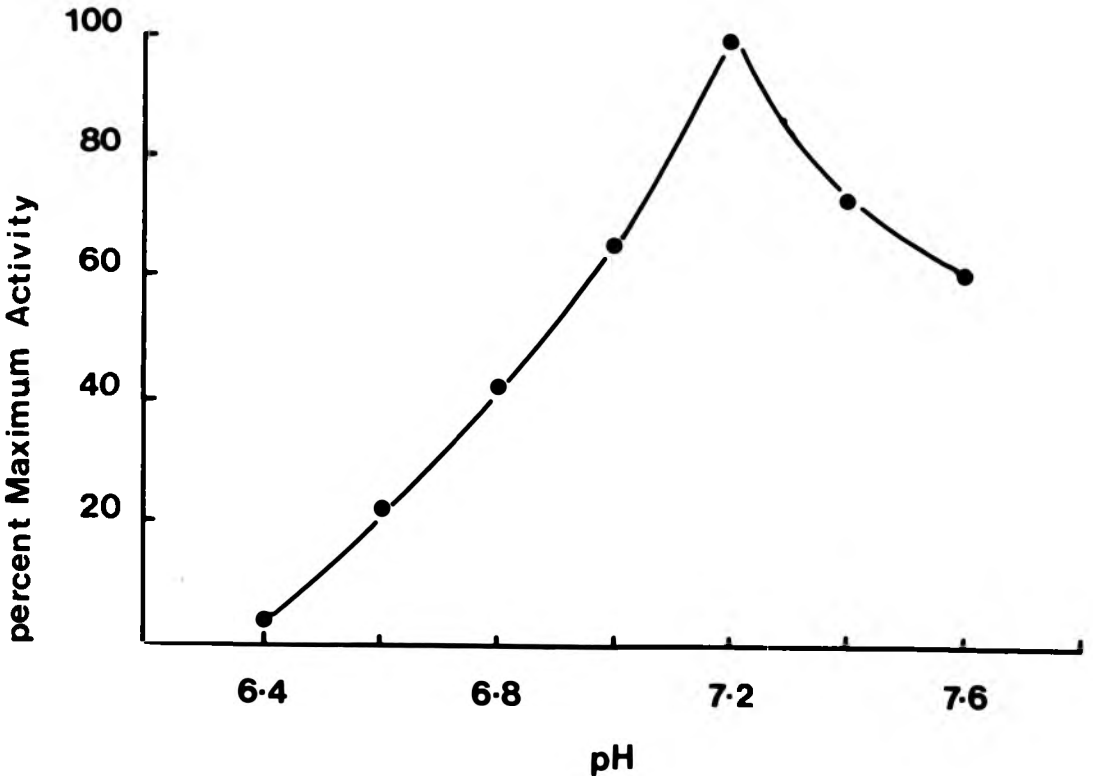

100
80
60
40
20
percent Maximum Activity
6·4
6·8
7·2
7·6
pH

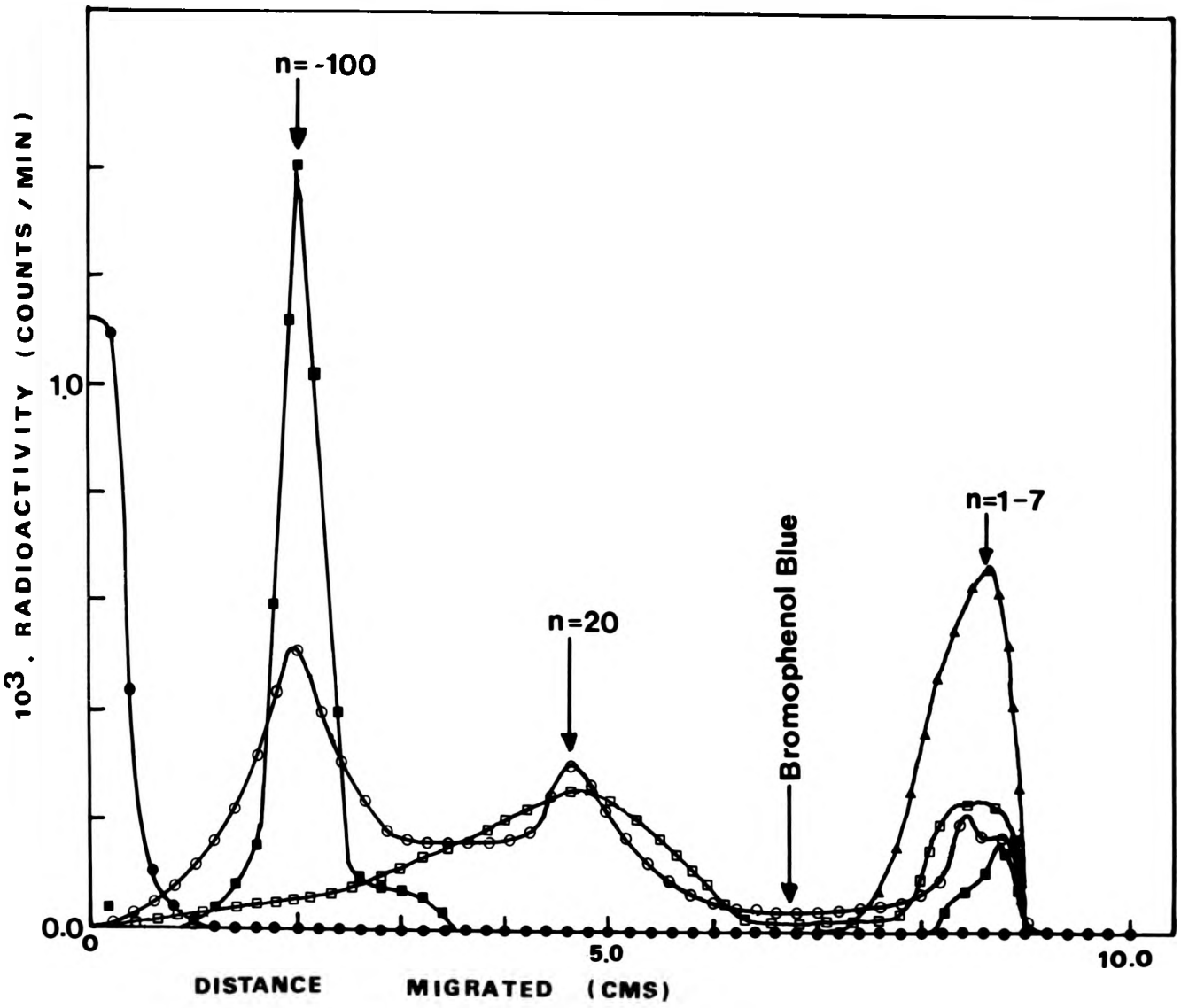
FIGURE 40

Gel electrophoresis of poly (A) hydrolysis products [^{14}C] - poly(A) (50 µl, 5 mg/ml) in 0.05 M MOPS pH 7.0 containing 2 mM magnesium chloride was incubated with enzyme (10 µl, 32 u/ml) at 37°. At given time intervals, aliquots were mixed with 50% sucrose and applied to 7.5% polyacrylamide gels containing 7 M urea. The gels were run in a tris-borate buffer pH 8.3 containing 7 M urea at 100 V and 5 mA per tube. The gels were then fractionated into 0.20 cm lengths, dried and dissolved in NCS tissue solubilizer (1.0 ml). The samples were counted in a toluene based scintillation fluid.

—●— 0 hours —□— 2 hours

—▣— ½ hour —▲— 24 hours

—○— 1 hour


n= ~100
n=20
Bromophenol Blue
n=1-7
10^3. RADIOACTIVITY (COUNTS / MIN)
1.0
0.0
0
5.0
10.0
DISTANCE MIGRATED (CMS)

is competitively inhibited by $d(pT)_3$ (Figure 41). The Ki for $d(pT)_3$ is 7.34×10^{-5} M while the Km for poly(A) under the same conditions is 7.7×10^{-5} M. $d(pT)_3$ does not interact with poly(A) under the conditions used (Cassani and Bollum, 1969) and the inhibition represents a true interaction between the enzyme and the oligodeoxynucleotide. Single stranded and double stranded DNA also act as inhibitors.

Even the purified enzyme exhibits a lag phase in the degradation of polymer when assayed by methods depending on the release of acid soluble products. The lag phase becomes more marked when the substrate polymer is preincubated with alkaline phosphatase to remove 5' terminal phosphate groups. Similar effects have been observed with a nuclease from Azotobacter agilis (Stevens and Hilmoe, 1960) which cleaved oligonucleotides lacking a 5' phosphomonoester group at less than 10% of the rate observed when the substrate had a 5' terminal phosphate group.

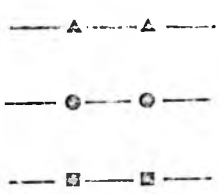
Properties of the nuclease Sepharose conjugate

The enzyme was successfully coupled to cyanogen bromide activated Sepharose, yielding a conjugate with a protein content of 14.5 mg/g matrix which represented a binding yield of 80%. The enzyme was coupled to the activated matrix (Axen and Ernback, 1971), but the instability of the enzyme at pH values above 8.0 made it necessary to couple the enzyme at the lower pH. The hydrolysis of poly(A) by a packed bed of the conjugate is shown in Figure 42.

FIGURE 41

Kinetics of poly(A) hydrolysis and competitive inhibition by $d(pT)_3$.

Assays were carried out in a total volume of 100 µl in 0.05 M MOPS, pH 7.0 containing 2 mM magnesium chloride as described in the Methods Section.



Poly (A)

Poly (A) + 3.725×10^{-5}M $d(pT)_3$.

Poly (A) + 7.45×10^{-5}M $d(pT)_3$.

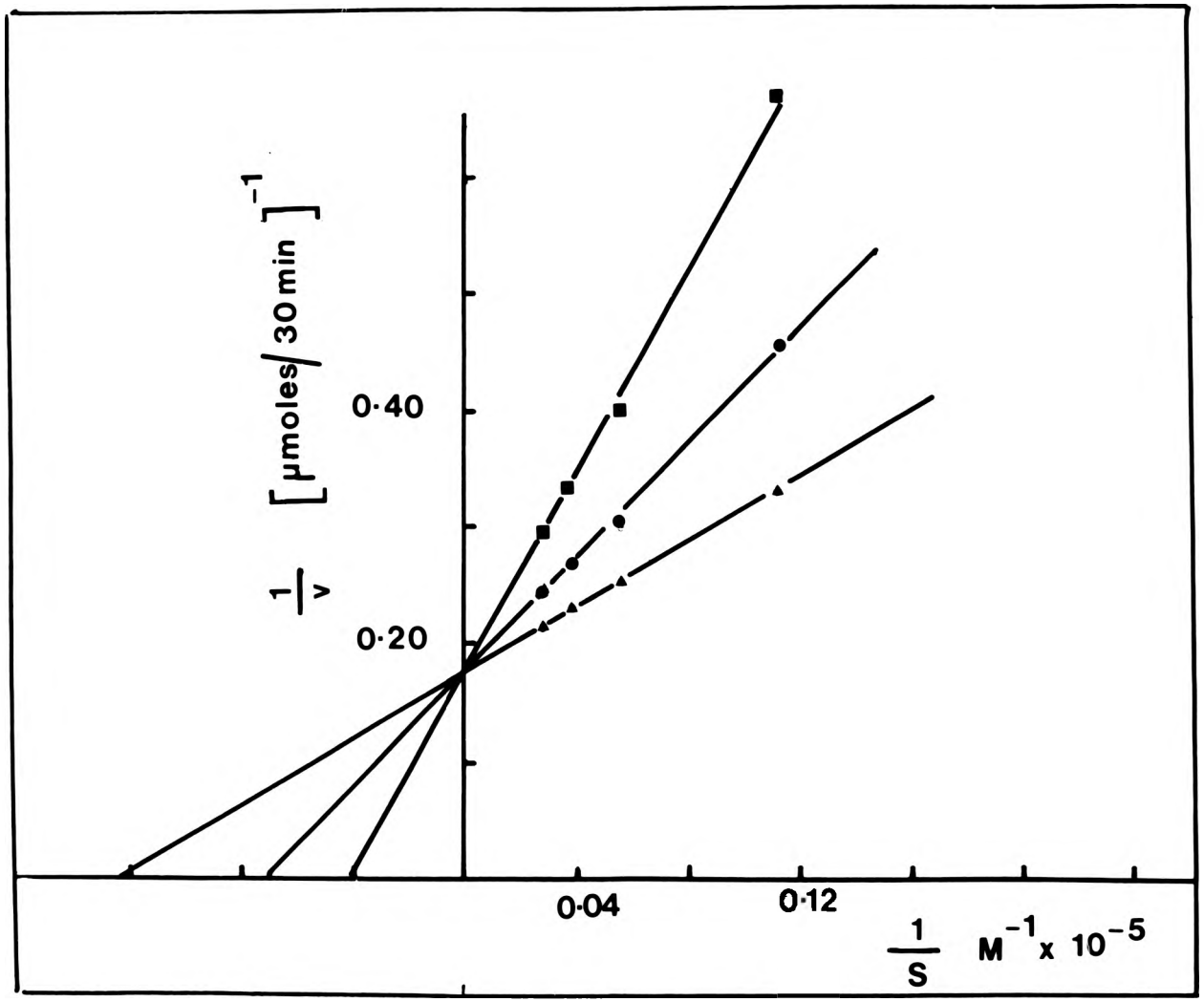

$\frac{1}{v}$ [µmoles/30 min]$^{-1}$
0·40
0·20
0·04
0·12
$\frac{1}{S}$ M^{-1} x 10^{-5}

FIGURE 42

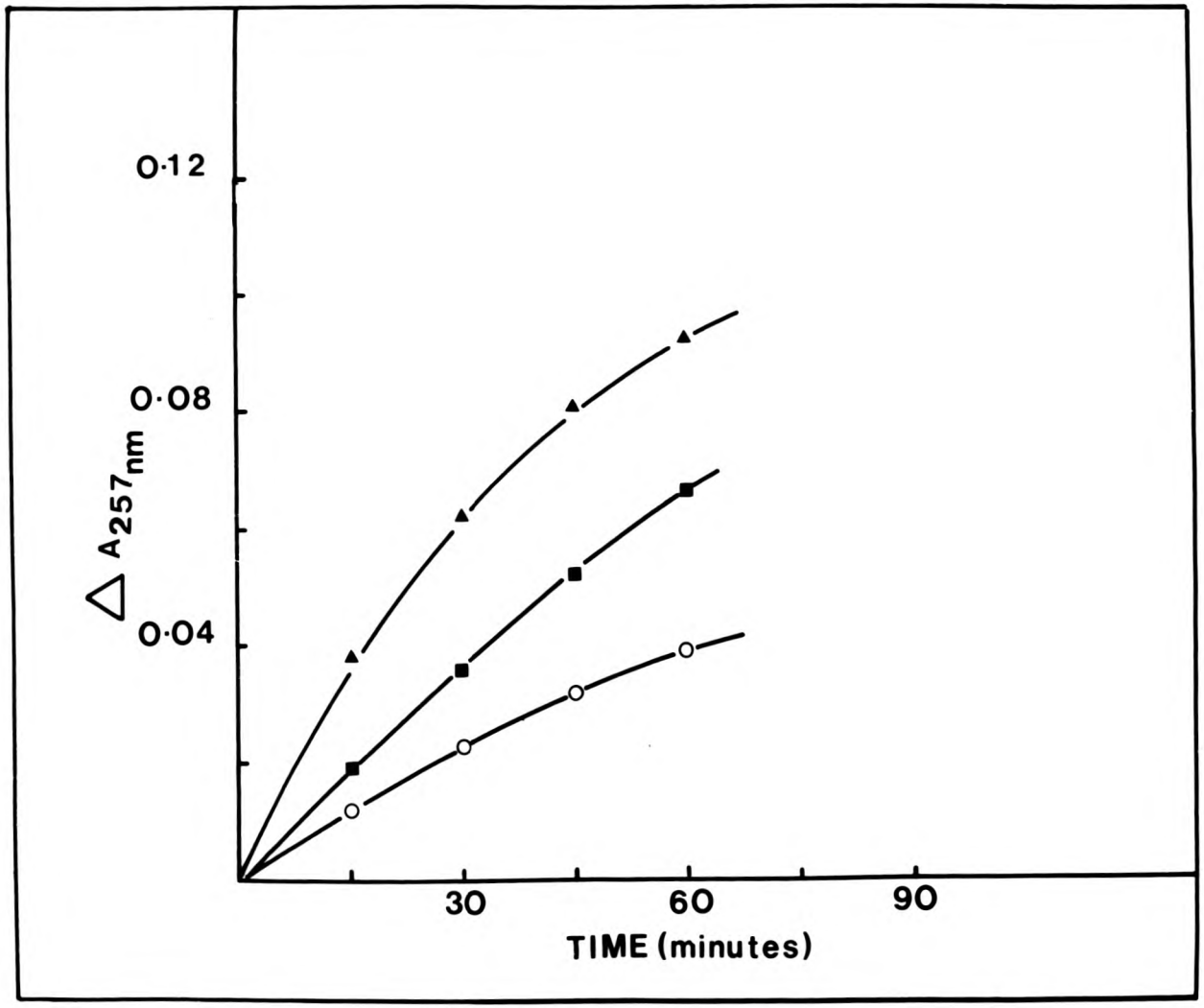
Recycling of poly(A) through a packed bed of immobilized nuclease.

A solution of poly(A) (15.0 ml) in 0.05 M MOPS, pH 7.0 containing 2 mM magnesium chloride was recycled through a packed bed of the nuclease-Sepharose conjugate at a flow rate of 1.0 ml min^{-1}. The reaction was followed by observing the increase in absorbance at 257 nm in 1 mm path length flow cells.

—— O —— O —— 1.6×10^{-3} M poly(A)

—— ■ —— ■ —— 2.4×10^{-3} M poly(A)

—— ▲ —— ▲ —— 3.2×10^{-3} M poly(A)


0·12
0·08
0·04
ΔA_{257nm}
30
60
90
TIME (minutes)

References

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