Biochimica et Biophysica Acta, 1000 (1989) 59–64
Elsevier

Recollections on studies of polynucleotide phosphorylase: a commentary by

Marianne Grunberg-Manago

Institute de Biologie Physico-chimique, Paris (France)

on 'Enzymic synthesis of polynucleotides. I. Polynucleotide phosphorylase of *Azobacter vinelandii*'
by M. Grunberg-Manago, P.J. Ortiz and S. Ochoa
Biochim. Biophys. Acta 20 (1956) 269–285

A. History of the discovery

When I arrived at New York University in the autumn of 1953 to work with Severo Ochoa, his group was concerned with the enzymatic reactions that were coupled to phosphorylation. In particular, Seymour Kaufman had discovered in spinach an enzyme referred to in the laboratory as P enzyme (named after succinic thiokinase) which catalyzes the synthesis of ATP from ADP + P and succinyl-CoA.

The mechanism of the P enzyme reaction was extensively investigated by studying exchange reactions between labelled $^{32}PO_4$ or ADP with ATP and labelled succinate with succinyl-CoA [1]. These studies revealed phosphorylated intermediates and led to the proposal of a detailed mechanism for the reaction. The method appeared very promising and Severo wanted to apply it to other reactions linked to phosphorylation. In particular, he thought the time was ripe to study what appeared to be the most crucial and fundamental problem of the moment: the synthesis of ATP coupled to oxidation: 'oxidative phosphorylation'. Most of the prestigious and often large groups (D. Green, P. Boyer, A. Lehninger, H. Lardy, E. Racker, M. Cohn) were competing to understand the mechanism of the phosphorylation coupled to electron transport. The enzymology of nucleic acids was being investigated elsewhere by quite small groups often trained in England (R. Markam, N. Pirie) or in Denmark (H. Kalkar). I don't think I ever heard in my first year as a post-doc at New York University the words 'nucleic acid'.

But before proposing 'the golden subject' (oxidative phosphorylation) to the new post-docs (Ernie Rose, from Chicago, and myself) who had arrived in his laboratory, Severo had to test their ability to purify enzymes and study their enzymatic mechanisms. He therefore proposed to us, as a start, another problem that he thought interesting: the mechanism of phosphorylation by acetokinase of acetate (without a CoA intermediate) to acetyl PO_4. He gave us a bottle containing dry *E. coli* cells and we understood we had to work out a purification procedure and a mechanism with the amount of cells in the bottle. I remember how worried we were watching the *E. coli* cells disappearing during our assays to establish a purification procedure [2]. In spite of the fact that the mechanism of acetokinase didn't excite us too much, the purification was good

Marianne Grunberg-Manago

0006-3002/89/$03.50

training for both of us and the enzyme turned out to be quite useful: on a suggestion of Terry Stadtmann who was visiting the laboratory, I worked out a procedure for the determination of acetate with acetokinase and Terry still uses this enzyme for some of her studies.

In fact, I remember with pleasure this period of collaboration with Ernie Rose. We both passed successfully the probationary period and, around Christmas, Severo proposed to us "the dearest project of his heart": oxidative phosphorylation. Ernie Rose chose to study it in rat mitochondria: he was quite excited by P. Boyer's and Mildred Cohn's studies with ^{18}O exchange and decided to use this method in mitochondrial studies. As I didn't like killing rats, I decided to study it in bacteria. I thought that, in choosing an obligatory aerobic bacterium like *Azotobacter vinelandi* which actively oxidizes carbohydrate, I would have a better chance of isolating an active system for ATP synthesis linked to oxidation. Realizing that it would be difficult to see a net PO_4 uptake in bacterial extracts contaminated with phosphatases and many other reactions consuming or releasing PO_4, I decided to use an exchange reaction between PO_4 and ATP as a method (experienced during acetokinase studies) to isolate some interesting new phosphorylated coenzymes. The idea, which now seems naïve, was that the reaction would be quite simple, involving a soluble coenzyme X which would be phosphorylated during ATP synthesis by a specific enzyme:

$$ATP + X \leftrightharpoons XP + ADP \rightleftharpoons ADP + P + X$$

I indeed observed an exchange of $^{32}PO_4$ with the two terminal phosphates of ATP in the soluble extract of *A. vinelandi* and I started purifying the protein responsible for the exchange. I used as substrate commercial amorphous ATP. During this work, the firm Sigma advertised a very pure crystalline ATP which they had just prepared. I managed to obtain some and, to my surprise, I no longer observed any exchange in the presence of the crystalline derivative with my partially purified protein fraction. This made Severo and myself quite happy, as we hoped that the previous amorphous preparation was contaminated with an interesting cofactor. I decided to isolate from the amorphous ATP preparation a fraction which, when added to crystalline ATP, would restore the exchange. To my surprise, the chromatographic examination of the amorphous ATP identified the active component as ADP. In fact, ATP was only labelled due to adenylkinase still contaminating the protein fraction (it is known that it is very difficult to get rid of traces of adenylate kinase).

I remember that when I recounted this discovery at a lunch group, nobody believed me and Severo offended me by saving that it was impossible. However, a moment later, regretting his first reaction, he came to the lab and I could easily convince him that the real substrate in the exchange reaction was ADP. He got very excited, as no known enzyme was able to catalyze such an exchange, and he encouraged me to pursue this and try to find out what was the reaction responsible for such an exchange. I soon found that the enzyme was not specific for adenosine diphosphate, but catalyzed the exchange with other diphosphate nucleotides (UDP, CDP, GDP and IDP).

But after the first excitment of discovering a new reaction, I had for a few months a very frustrating period. I purified the enzyme some more but still couldn't identify the reaction; there was a slight PO_4 liberation during exchange, but that might have been ascribed to some residual phosphatase contamination. Severo also began to be discouraged, particularly because at the same time G. Pinchot had isolated from *Alcaligenes faecalis* different fractions which, when mixed, catalyzed a net PO_4 uptake coupled to electron transport [3]. Severo started doubting the meaning of the exchange and encouraged me to try G. Pinchot's reconstitution experiment with *Azobacter* extracts. I remember that G. Pinchot visited us and did some experiments in the lab. However, I was not prepared to drop my approach straightaway. In particular, I was puzzled by the slight PO_4 liberation, as I felt the enzyme was well purified from phosphatases and I decided to investigate the reason for this liberation.

At that time, Severo was leaving for Europe (I think for some kind of business related to the International Union of Biochemistry). I promised him that if I had no results on his return, I would start de novo looking for PO_4 uptake in *Azobacter* extracts. I did a very simple experiment which was decisive for the discovery of polynucleotide phosphorylase. I replaced ADP by inosine diphosphate; adenylate kinase is inactive with the inosine derivative and I thereby avoided the complications of mono- and tri- derivatives formed by adenylate kinase. I performed a saturation curve (one has to realise that the diphosphates were not easily available and were expensive and I had to justify using so much diphosphate for an experiment which appeared trivial). However, under saturation conditions (polynucleotide phosphorylase has a low affinity for the diphosphate derivatives), I observed a large amount of PO_4 liberation. It was a relief to find that I was dealing not only with an exchange reaction, but also with a reaction during which PO_4 was liberated. I immediately started identifying the other product of the reaction by chromatography. At this point, it might have been a rather uninteresting reaction, namely a hydrolysis of diphosphates to monophosphates. Coincidentally, in the department G. Plaut was studying an IDPase he had isolated from rat liver mitochondria [4]; however, the reaction in my case appeared to be reversible, and the reversibility of a hydrolytic reaction seemed very unlikely. Chromatography of the reaction mixture on a

Marianne Grunberg-Manago with Severo Ochoa (right) and her husband (left) at an exhibition of the latter's work

Dowex column revealed no IMP formed, which made me very happy. But at first, I couldn't identify in the eluate from the column any new product. It was therefore possible that the enzyme synthesized a compound which was not eluted under my experimental conditions. I started to hope, without believing it completely, that the product which stuck to the column might be a high-molecular-weight compound. Fortunately, by using paper chromatography, I could identify in the reaction mixture after enzymatic incubation a new UV spot which didn't move from the origin of the chromatogram. I knew at that point that the enzyme had synthesized a polynucleotide. I will never forget the day I saw the new UV spot. I was so excited that I wanted to tell everybody in the lab, but to my disappointment nobody was there as it was some kind of holiday. In the end, I called Severo at home: he was wondering what had happened. He was certainly quite pleased with the discovery but was still hoping in the depth of his heart that the synthesized product had a pyrophosphate linkage and was involved in one way or another in oxidative phosphorylation, which shows how far from molecular biology was the preoccupation of enzymologists at that time. But soon afterwards, we all became excited by the discovery. Severo told me that, when giving a seminar on P enzyme at Bethesda, he mentioned the discovery at the end (we didn't yet know the structure of the polymer), and observed H. Kalkar, who had been dozing as usual during the seminar, jump up in his chair all awake!

Through the help of friends like L. Heppel, J. Fresco and A. Rich, the results came very quickly. I could show that the product was precipitated by acid (that was another exciting moment when I saw the polymer form a solid gel), discovered that the polyl had a high molecular weight (A. Rich did the first determination of the molecular weight) and found that the polymer had a diester linkage. L Heppel had all the necessary enzymes needed to identify the structure and he gave us with his accustomed generosity all we needed. Using a mixture of adenosine, uridine, cytosine and guanosine diphosphates, I was able to synthesize an RNA-like copolymer with the four bases.

We had some discussion as to how to name the enzyme. Severo was hoping that in vivo it might be involved in some way (maybe in the presence of a primer) in polynucleotide synthesis and was inclined to call it RNA synthetase, while I thought it would be safer to call it polynucleotide phosphorylase: finally, this name was adopted (Ref. 5 and this paper).

I presented the work in the spring of 1955 at the Meeting of the Federation of Societies for Experimental Biology in San Francisco. I remember that the big hall was quite empty but that, just before my presentation, it became jammed with people (the rumour had spread). The discovery aroused considerable interest; it was the first time that high-molecular-weight, RNA-like polynucleotides had been synthesized outside the cell and it encouraged the search for enzymes responsible for nucleic acid synthesis.

B. Seen in retrospect: what role did the discovery of polynucleotide phosphorylase play?

The greatest impact of polynucleotide phosphorylase was due to its ability to synthesize polymers and oligonucleotides. Polynucleotide phosphorylase has no specificity for the bases: numerous homopolymers, copolymers of natural or base analogues have been synthesized [6–8] which made possible many studies of fundamental properties of nucleic acids and nucleases. I had so many requests that for a few years I became a polymer factory. Thanks to Sigma, which provided diphosphates free, I was able to synthesize all kinds of homo- and copolymers and send them all over the world. When I was still at New York University, I recall giving to Jacques Fresco, who was working in the laboratory a floor below mine, some poly(A) and poly(U). Jacques mixed them and I was present at what I think was the first demonstration of the poly(A)–poly-(U) interaction: as if by magic, clear solutions of poly(A) and poly(U) were transformed into a solid gel. Jacques turned the tube upside down; not a drop came out.

The double-stranded structure of poly(A) and poly(U) was soon established by Alex Rich through X-ray diffraction analysis and was thought to be similar to that of DNA (the structure of which had been published by J. Watson and F. Crick not too long before in 1953). This created considerable interest and I spent some time in P. Doty's lab working with J. Fresco. The excitment of annealing complementary strands was great and was explored extensively and so efficiently by the groups of J. Marmur, P. Doty and J. Fresco. This led to establishment of modern hybridization techniques. The model of RNA structure (hairpin and loop structure) was also established some time later by the group of P. Doty [9,10], using as a model the interaction of mismatched copolymers. The more recent structure of ribosomal RNA confirms their original model.

While polynucleotide phosphorylase has no specificity for bases, it has, however, a high specificity for the sugar (ribo versus deoxy). But special conditions were found that enabled the enzyme to synthesize oligodeoxynucleotides of defined sequence [11]. The enzyme has also a specificity for the structure *syn-anti* and shows a sensitivity towards secondary structure. Thus, single-stranded RNA is readily phosphorylized, whereas double- or triple-stranded structures are largely resistant [12]. I was able to use the enzyme to study polynucleotide structure [12,13], in particular that of tRNA. I was fortunate to collaborate with M. Cohn, and interesting effects of metal ions [14] on the tRNA

structure were found. We also made a curious observation that is still not well understood [13]. At 37°C, polynucleotide phosphorylase phosphorylizes only some of the tRNA molecules present in the reaction mixture, whereas the remaining chains appear to be completely intact (not even the terminal A is removed). To phosphorylize all RNA chains by the enzyme, the temperature of the reaction mixture has to be elevated to 45°C to permit a configurational change of the tRNA. The work on tRNA allowed me to collaborate with Mildred Cohn, who spent her sabbatical in my laboratory and started a friendship which I still enjoy.

The structure and the mechanism of polynucleotide phosphorylase were investigated by three groups, M. Singer with L. Heppel and later with C. Klee, U. Littauer and ourselves. These two last groups worked mostly with the *E. coli* enzyme (with for us a few incursions into *C. perfringens*), while M. Singer and C. Kloe worked mainly with *M. luteus* [6,7]. There was no competition between the different groups, but a complementary research, and from that time began a long friendship between myself and M. Singer, U. Littauer and L. Heppel. I remember spending some time in Bethesda working with Maxine and sharing a bench with G. Khorana, who also happened to spend some time with L. Heppel. The structures and mechanisms of the *E. coli* and *M. luteus* phosphorylases looked different at first, but in fact their structure and properties are very similar. The difference which was found to be quite useful was due to proteolysis. The *M. luteus* enzyme, because of a high amount of contaminating proteinases, is usually purified in a proteolyzed form, has an obligatory primer requirement and exhibits a synchronous mechanism of degradation or polymerization. Therefore, it was extensively used to synthesize oligonucleotide of defined structure, in particular by R. Thach for investigations on protein synthesis and by O. Uhlenbeck for studies on RNA–protein interactions. In contrast, the intact *E. coli* or *M. luteus* enzymes are able to initiate synthesis de novo without a primer requirement and they degrade polymers or polymerize diphosphates by a processive mechanism. The structure of the enzyme (a trimer) [15] and the mechanism of polymerization and phosphorolysis were quite novel at that time. To account for the non-classic behaviour of *E. coli* polynucleotide phosphorylase, a model has been proposed. In addition to a catalytic site which attacks the 3′ end of a polymer or oligonucleotide, there exists a second group of attachment points which are reached when the polymer is long enough. They contribute to the stability of the enzyme-polymer complex [6,7]. Interestingly enough, there are primary structure homologies between a 69-amino-acid stretch of polynucleotide phosphorylase and the four homologous stretches of ribosomal protein S1 which form its RNA binding site. The possibility is considered that this 69-amino-acid stretch constitutes the polynucleotide binding domain of polynucleotide phosphorylase [16].

It is clear now that the enzyme is not responsible for the synthesis of RNA in vivo. It is also clear that, in parallel with another nuclease RNase II, it has an important function in vivo. The double mutation in PNPase gene, pnp^- and in RNase II gene, rnb^- is lethal in *E. coli*, but when the mutant strain is supplemented with a plasmid carrying the PNPase gene (*pnp*), growth is restored [17]. The strains $rnb^+ - pnp^-$ and $rnb^- - pnp^+$ are able to grow. The fact that the cell lacking either RNase II or PNPase does not show a defect in growth suggests the two enzymes have the same function and indicates that PNPase is probably involved in mRNA degradation.

The genetics of polynucleotide phosphorylase also gave some surprises: the polynucleotide phosphorylase gene is part of a complex operon containing the gene *rpsO* for ribosomal protein S15 and is situated in a cluster of genes containing translation initiation factor IF2 that, by coincidence, we were extensively studying in our group. A peculiarity of the operon containing the polynucleotide phosphorylase gene is that between the promoter and the polynucleotide phosphorylase structural gene there exists an RNase III processing site, responsible for the instability of polynucleotide phosphorylase mRNA in wild-type strains. In RNase III gene, rnc^- mutants [18], the mRNA is unusually stable, emphasizing the rôle of RNase III in messenger RNA stability.

Finally, above all is the use of polynucleotide phosphorylase in the early deciphering of the genetic code. The discovery of M. Nirenberg and J. Matthai [19] that a system from *E. coli* translated poly(U) into polyphenylalanine was the most exciting news I heard at the International Congress in Moscow (1961), and it became clear that this opened an experimental approach to the study of the coding problem in protein biosynthesis. In the following month, a race started between the group of Nirenberg (who could get the polymers from M. Singer) and the group of S. Ochoa, on the effect of a large variety of copolymers on amino-acid incorporation. The compositions of many codons were very quickly found. I remember the excitment of this period, the communication of results by telephone, some notes in the laboratory which for a nonscientist (as my husband) looked strange, who noted: "The code is completely degenerate". I didn't think at first to join this race, as I was working alone with a technician. However, in discussion with F. Crick and M. Bretscher (Cambridge), we were puzzled that all the codons found to date contained uracil. It was believed at that time that there was something magic about uracil and few different explanations had been proposed. It seemed unreasonable to us that messenger RNA would have such a high proportion of uracil, given the base com-

position of DNA. So we thought that the finding that only polymers containing uracil were active was purely technical, due to the isolation of polypeptides, and we decided to try the coding capacity of copolymers containing only C, A or G in different proportions and we were quite pleased to find them active in promoting the incorporation of histidine, threonine and asparagine. We could report the composition of the codon for threonine (ACC, ACA) histidine (CAC) and asparagine (AAC) [20].

Our second contribution was to show that there occur errors in the translation of the genetic code. We had shown for the first time that the incorporation of amino acids in presence of poly(U) was not limited to phenylalanine, but that different amounts of leucine were also incorporated. We checked very carefully in all these studies that the observed incorporation was not due to amino-acid or diphosphate impurities [20]. After that I studied, in collaboration with M. Michelson as well as a variety of other workers, the problem of error in translation, particularly in the presence of different analogues [21]. During that time the deciphering of the genetic code was stimulated by the discovery of M. Nirenberg and P. Leder that a triplet promotes the binding to ribosomes of specific aminoacyl-tRNAs. They, together with G. Khorana and D. Söll, synthesized very quickly all the triplets and determined the base sequence of all the codons. I was visiting NIH just before the International Congress in New York and Marshall (Nirenberg) and Phil (Leder) invited Maxine and myself to lunch and told us about their discovery. I remember my enthusiasm about their work. The genetic code also was confirmed by G. Khorana, who synthesized polymers of defined base sequence.

After the incursion into the genetic code, I continued for some time to be in contact with the Cambridge group, composed at this time of A. Smith, B. Clark and K. Marcker, who also worked on the mechanism of initiation of protein synthesis. I remember that period with particular pleasure. I enjoyed getting to know better the Cambridge group and F. Crick. I liked his enthusiasm, simplicity in discussing in the small groups, and wisdom.

Polynucleotide phosphorylase has brought a lot of pleasure to me. It was the key to the development of modern molecular biology and its importance was recognized by the Nobel Prize in 1959.

Correspondence: M. Grunberg-Manago, Institute de Biologie Physico-Chimique, rue P. et M. Curie, F 75005 Paris, France.

References

1 Kaufman, S. (1955) J. Biol. Chem. 216, 153–164.
2 Rose, I.A., Grunberg-Manago, M., Korey, S.R. and Ochoa, S. (1954) J. Biol. Chem. 211, 737–756.
3 Gifford, B. and Pinchot, G.B. (1953) J. Biol. Chem. 205, 63–74.
4 Plaut, G.W.E. (1955) J. Biol. Chem. 217, 235–245.
5 Grunberg-Manago, M. and Ochoa, S. (1955) J. Am. Chem. Soc. 77, 3165–3166.
6 Godefroy-Colburn, T. and Grunberg-Manago, M. (1972) Polynucleotide Phosphorylase. The Enzymes, Vol. VII, pp. 533–574, Academic Press, New York.
7 Littauer, U.Z. and Soreq, H. (1982) Polynucleotide Phosphorylase, The Enzymes, Vol. XV, PP. 517–533, Academic Press New York.
8 Godefroy-Colburn, T. (1976) Synthesis of nucleic acid by polynucleotide phosphorylase, Methodicum Chemicum (Korte, Goto and Mazuren, eds.), Vol. 11, Part 1, pp. 42–54, Academic Press, New York.
9 Doty, P., Boedtker, H., Fresco, J.R., Haselkorn, R. and Litt, H. (1959) Proc. Natl. Acad. Sci. USA 45, 482–498.
10 Fresco, J.R., Alberts, B.M. and Doty, P. (1960) Nature 188, 98–101.
11 Gillam, S., Rottmann, F., Jahnke, P. and Smith, M. (1977) Proc. Natl. Acad. Sci. USA 74, 96–100.
12 Grunberg-Manago, M. (1959) J. Mol. Biol. 1, 240–259.
13 Thang, M.N., Guschlbauer, W., Zachau, H. and Grunberg-Manago, M. (1967) J. Mol. Biol. 26, 403–421.
14 Cohn, M., Danchin, A. and Grunberg-Manago, M. (1969) J. Mol. Biol. 39, 199–217.
15 Portier, C. (1975) FEBS Lett. 50, 79–81.
16 Régnier, P., Grunberg-Manago, M. and Portier, C. (1987) J. Biol. Chem. 262, 63–68.
17 Donovan, W.P. and Kushner, S.R. (1986) Proc. Natl. Acad. Sci. USA 83, 120–124.
18 Portier, C., Dondon, L., Grunberg-Manago, M. and Régnier, P. (1987) EMBO J. 6, 2165–2170.
19 Nirenberg, M.W. and Matthai, J.H. (1961) Proc. Natl. Acad. Sci. USA 47, 1588–1602.
20 Bretscher, M. and Grunberg-Manago, M. (1962) Nature 195, 283–284.
21 Grunberg-Manago, M. and Michelson A.M. (1964) Biochim. Biophys. Acta 80, 431–440.

ENZYMIC SYNTHESIS OF POLYNUCLEOTIDES

I. POLYNUCLEOTIDE PHOSPHORYLASE OF *AZOTOBACTER VINELANDII**

by

MARIANNE GRUNBERG-MANAGO**, PRISCILLA J. ORTIZ AND SEVERO OCHOA

Department of Biochemistry, New York University College of Medicine, New York, N.Y. (U.S.A.)

As previously reported[1,2], an enzyme isolated from the microorganism *Azotobacter vinelandii* catalyzes the synthesis of highly polymerized polynucleotides from 5'-nucleoside diphosphates with release of orthophosphate. The reaction, which requires magnesium ions and is reversible, can be represented as in Equation (1) where R stands for ribose, P-P for pyrophosphate, P for orthophosphate, and X for one or more of the following bases: adenine, hypoxanthine, guanine, uracil or cytosine.

$$n\text{X–R–P–P} \rightleftharpoons (\text{X–R–P})_n + n\text{P} \qquad (1)$$

The reaction is analogous to the reversible synthesis and breakdown of polysaccharides catalyzed by phosphorylase and, for this reason, the name polynucleotide phosphorylase has been proposed[1] for the new enzyme. Chemical and enzymic degradation of the biosynthetic polynucleotides, carried out in part in collaboration with Dr. LEON A. HEPPEL of the National Institutes of Health, has shown that they are made up of 5'-nucleoside monophosphate units linked to one another through 3'-phosphoribose ester bonds as in ribonucleic acid[2]. It has further been shown[2] that, from a mixture of ADP***, GDP, UDP and CDP, the *Azotobacter* enzyme catalyzes the synthesis of polynucleotides which, in the light of chemical and enzymic evidence, cannot be distinguished from natural RNA.

Recent experiments indicate that polynucleotide phosphorylase is widely distributed and suggest that this enzyme may be essential for the synthesis and

* This work was aided by grants from the National Institute of Arthritis and Metabolic Diseases (grant A-529) of the National Institutes of Health, U.S. Public Health Service; The American Cancer Society (recommended by the Committee on Growth, National Research Council); the Rockefeller Foundation; and by a contract (N6 onr 279. T.O.6) between the Office of Naval Research and New York University College of Medicine.

** Present address: Institut de Biologie Physico-Chimique, 13, rue Pierre Curie, Paris Ve, France.

*** The following abbreviations are used: Ribonucleic acid, RNA; deoxyribonucleic acid, DNA; 5'-diphosphates (pyrophosphates) of adenosine, inosine, guanosine, uridine and cytidine, ADP, IDP, GDP, UDP and CDP; 5'-monophosphates of the same nucleosides, AMP, IMP, GMP, UMP and CMP; adenosine- and inosine-5'-triphosphate, ATP and ITP; ultraviolet, U.V.; counts per minute, c.p.m.; ethylenediaminetetraacetic acid (versene), EDTA; and tris(hydroxymethyl)-aminomethane, Tris.

References p. 285.

breakdown of RNA in the cell. In this connection it is of interest that POTTER and his collaborators[3] have demonstrated the occurrence in tissues of 5'-mono-, di-, and triphosphates of adenosine, guanosine, uridine and cytidine. POTTER[4] has recently suggested the nucleoside diphosphates as the most likely precursors of RNA.

The partial purification of the *Azotobacter* enzyme, the properties of the system, and the preparation and isolation of various polynucleotides, are described in this paper. Other papers of this series will be concerned with further studies of the structure of biosynthetic polynucleotides, their physical properties, the distribution and the isolation of polynucleotide phosphorylase from various sources.

EXPERIMENTAL

Preliminary experiments

Polynucleotide phosphorylase was discovered in the course of a study of biological phosphorylation mechanisms[5]. The aim of this work was to look for soluble enzyme systems catalyzing the incorporation of radioactive orthophosphate in ATP. On incubation of dialyzed *Azotobacter* extracts with ATP, in the presence of Mg^{++}, there was a rapid "exchange" or incorporation of the labeled phosphate. Since the extracts contained adenylic kinase (myokinase)[6], the labeled phosphate was incorporated both in ATP and ADP. The early experiments were carried out with amorphous preparations of ATP which contained ADP as a contaminant. However, when crystalline ATP was used there was little or no "exchange". Eventually, it was found that the "exchange" was promoted by ADP, prepared from crystalline ATP through reaction with glucose in the presence of hexokinase, and not all by ATP or AMP. It was

TABLE I

PHOSPHATE "EXCHANGE" AND LIBERATION WITH VARIOUS NUCLEOTIDES

*Nucleotide**	*Purity*	*P incorporation in 15 min c.p.m.*	*P liberation in 30 min μmoles*
AMP	Crystalline	0	0
ATP	Amorphous	5000	
ATP	Crystalline	256	0.07
ADP	Amorphous	41000	0.35
ADP	Crystalline**	41000	0.35
ADP + boiled extract***	Amorphous	42000	
ATP + AMP	Crystalline	17200	0.20
ADP (no Mg^{++})	Amorphous	0	0
ITP	Amorphous	195	0.06
IDP	Amorphous	57000	0.52

Samples contained (in μmoles), Tris buffer, 100; $MgCl_2$, 10; and EDTA, 1. Final volume, 1.0 ml; incubation at 30°. In addition, the samples for phosphate incorporation (pH, 8.1) contained potassium phosphate buffer, 2.3, with ^{32}P-labelled orthophosphate (142,000 c.p.m.); nucleotide as indicated, 3; and enzyme of specific activity 1.94 (assay 1) with 0.83 mg of protein (1.6 units). The samples for phosphate liberation (pH, 7.4) contained nucleotide as indicated, 5; and enzyme of specific activity 1.7 (assay 1) with 0.35 mg of protein (0.6 unit).

* All nucleotides are the 5'-mono- or polyphosphates.

** Prepared from crystalline ATP by reaction with glucose in the presence of crystalline hexokinase, and isolation of the ADP by ion-exchange chromatography[7].

*** Initial *Azotobacter* extract (9.7 mg of protein per ml) heated for 3 minutes at 100°. 0.2 ml of supernatant fluid used for experiment.

References p. 285.

further found that nucleoside diphosphates other than ADP, for example IDP, promoted the "exchange" reaction and that, simultaneously with the incorporation of labeled phosphate, there was a liberation of orthophosphate (*cf.* Reaction 1) which also required magnesium ions. These experiments were performed with enzyme preparations in which the activity had been partially purified employing the "exchange" with ADP as an assay.

Typical data with partially purified enzyme preparations are shown in Table I. With the exception of amorphous ATP, only the nucleoside diphosphates were active in giving rise to either incorporation of phosphate or phosphate liberation; the mono- and triphosphates had little or no activity. Positive results were also obtained with ATP plus AMP owing to the presence of adenylic kinase. This enzyme catalyzes the reaction ATP + AMP $\rightleftharpoons$ 2ADP. Similar results are shown in Fig. 1. These experiments were carried out with an enzyme preparation largely freed of adenylic kinase by precipitation with acetone. After incubation of the nucleotides with the enzyme, in the presence of Mg^{++}, and deproteinization of the reaction mixture the nucleotides were separated by paper chromatography in the solvent system of KREBS AND HEMS[8] followed by autoradiography to localize the radioactive spots containing ^{32}P. It will be seen that on incubation of mixtures of ADP or IDP with other nucleotides only ADP and IDP became labeled. The fact that AMP was not labeled when ADP and AMP were incubated together indicated that the radioactive phosphate was incorporated in the terminal phosphate of ADP. On incubation of IDP and ADP together both became labeled although, in this experiment, the labeling of ADP was weak. Fig. 2 shows the time course of incorporation of ^{32}P-orthophosphate in ADP as well as that of liberation of orthophosphate from ADP.

A number of experiments were carried out with IDP as substrate to determine the stoichiometry of the reaction. IDP, rather than ADP, was used because adenylic kinase is not active on IDP. When the incubated reaction mixtures were deproteinized

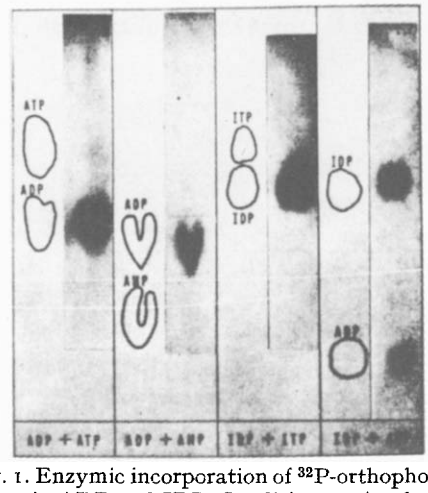

Fig. 1. Enzymic incorporation of ^{32}P-orthophosphate in ADP and IDP. Conditions as in phosphate incorporation experiments of Table I. Paper chromatography by the method of KREBS AND HEMS[8]. Spots located with U.V. lamp. Corresponding autoradiograms to the right of sketch of each chromatographic strip.

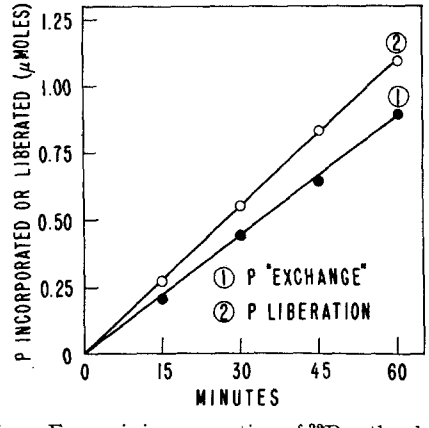

Fig. 2. Enzymic incorporation of ^{32}P-orthophosphate in ADP (Curve 1) and liberation of orthophosphate from ADP (Curve 2) as a function of time. The conditions were those of the standard assays described in the text. 20.5 μg of enzyme of specific activity 10.6 (0.218 unit) were used for Curve 1, and 61.6 μg of enzyme of specific activity 13.0 (0.8 unit) for Curve 2. The specific activities are expressed in terms of assay 1.

References p. 285.

with trichloroacetic acid, there was a disappearance of total nucleotide (determined by measurement of U.V. absorption) from the supernatant fluid corresponding to the amount of IDP disappearing and that of orthophosphate liberated. On the other hand, there was no disappearance of total nucleotide when the reaction mixture was deproteinized by heating for 1 or 2 minutes at 100° (Table II). However, the reaction product could not be eluted from Dowex-1 (formate) columns with formic acid at concentrations much higher than those required to elute the most acidic mononucleotides. A typical experiment is shown in Table II. In this experiment 2.0 ml aliquots of the protein-free supernatant fluid were used for the separation of nucleotides by ion-exchange chromatography. The samples were brought to 50 ml with water and made alkaline by addition of 0.5 ml of concentrated ammonia. The solution was chromatographed on a Dowex-1 (formate) column (4 × 1 cm). The resin used was Nalcite SDR, 200–400 mesh, 2% cross-linkage. After placing the sample on the column, it was washed with water. Then it was eluted successively with 0.1 *M* sodium formate (which removes the orthophosphate), 0.15 *M* sodium formate with 0.01 *M* formic acid (which elutes the IMP), 0.2–0.3 *M* sodium formate with 0.01 *M* formic acid (which elutes the IDP) and, finally, with 0.4 *M* sodium formate with 0.01 *M* formic acid (which elutes ITP). It may be seen in the table that, on incubation for 20 and 60 minutes, the disappearance of IDP and the liberation of orthophosphate were equimolar. It may also be seen from the Δ values of the last column that there was a deficit in the nucleotide recovered from the column of the same order of magnitude as the IDP disappearance and orthophosphate liberation. As already reported[2], it was further found that the product of the reaction of IDP or ADP, in the presence of the enzyme and magnesium, was non-dialyzable against distilled water or dilute salt solutions. It could be quantitatively precipitated with trichloroacetic acid or alcohol in the cold; either of these two methods could be used for its isolation. The product was soluble in water, yielding relatively viscous solutions with a typical nucleotide ultraviolet absorption spectrum, and it remained at the origin of paper chromatograms whichever the solvent system used. All these facts indicated that the product was a polynucleotide.

TABLE II

REACTION WITH IDP

Incubation min	*Orthophosphate*	*IMP*	*IDP*	*Nucleotide recovered from column*
0	1.32	0.6	10.7	11.3
20	3.80	0.6	8.6	9.2
Δ	+2.48	0	−2.1	−2.1
60	6.40	0.6	6.5	7.1
Δ	+5.08	0	−4.2	−4.2

The reaction mixture contained the following components (in μmoles per ml), Tris buffer, pH 8.1, 36; $MgCl_2$, 4.8; potassium phosphate, pH 8.1, 1.3; IDP, 11.5; and enzyme of specific activity 4.6 (assay 1) with 0.54 mg of protein (2.5 units). Final volume, 8.5 ml. 2.5 ml aliquots removed at time zero and after incubation for 20 and 60 minutes at 30°. After heating for 2 minutes at 100°, cooling, and removing the precipitated protein by centrifugation, aliquots of the supernatant fluid were taken for the determination of orthophosphate (0.1 ml) and nucleotides (2.0 ml). Values expressed in μmoles per ml of reaction mixture. The small amount of IMP was present as a contaminant of the IDP preparation. There was no change in the amount of total nucleotide (determined by U.V. absorption at wave-length 247 mμ) during incubation.

References p. 285.

Isolation of enzyme

Enzyme assays. The enzyme has been assayed in either of two ways: (1) by determining the rate of incorporation of radioactive orthophosphate into nucleoside diphosphates (phosphate "exchange" assay) and (2) by determining the rate of liberation of orthophosphate from nucleoside diphosphates. Either ADP or IDP, but mostly the former, has been used for assay purposes.

Assay 1. The reaction mixture contains the following components (in μmoles): Tris buffer, pH 8.1, 100; $MgCl_2$, 5; ADP (or IDP), 2.5; potassium phosphate, pH 8.1, with 100,000–200,000 c.p.m. ^{32}P, 3.6 (or 2.5); and enzyme. Final volume, 1.0 ml. If ADP is used, the molar ratio ADP to orthophosphate should be 0.7; if IDP is used, the ratio IDP to orthophosphate should be 1.0. It is convenient to add a small amount of EDTA for assay of the enzyme during the initial steps of the purification. The reaction mixture is incubated for 15 minutes at 30°. With small amounts of enzyme the reaction is linear for at least 60 minutes. One unit of enzyme is defined as the amount which catalyzes the incorporation of 1.0 μmole of $^{32}PO_4^{\equiv}$ under the conditions of the assay in 15 minutes at 30°. The radioactivity in the organic phosphate fraction, determined as described in the next section, is used to calculate the amount of orthophosphate incorporated from the expression:*

$$\mu\text{moles phosphate incorporated} = \frac{\text{c.p.m. incorporated } (\mu\text{moles phosphate} + \mu\text{moles ADP})}{\text{c.p.m. phosphate}}$$

The specific activity of the enzyme is expressed as units per mg of protein. Protein was determined either by the biuret method[9] with crystalline ovalbumin, previously dialyzed free of salt, as a standard, or spectrophotometrically by measuring the absorption of light at wave-lengths 280 and 260 mμ, with a correction for the nucleic acid content from the data given by WARBURG AND CHRISTIAN[10]. The first method was used to determine the protein content of the extract and the first ammonium sulfate fraction, with high nucleic acid content, the second method was used for the later steps in purification.

Determination of orthophosphate incorporation. This procedure follows that described by ROSE AND OCHOA[11] with some modifications. The orthophosphate incorporated is given by the radioactivity remaining in the protein-free filtrate after removal of the orthophosphate. The procedure is based on the quantitative conversion of orthophosphate to ammonium phosphomolybdate and the removal of the latter by extraction with *iso*butanol, according to BERENBLUM AND CHAIN[12]. The reaction is stopped by addition of 0.1 ml of 40% trichloracetic acid per 1.0 ml of reaction mixture. After centrifugation, aliquots are taken for determination of the specific radioactivity of the orthophosphate and the radioactivity fixed. For removal of the orthophosphate, usually 1.0 ml of the protein-free filtrate is made up with water to 3.0 ml. To this solution are added 0.3 ml of 10.0 *M* H_2SO_4, 1.5 ml of 5% ammonium molybdate, and 5.0 ml of *iso*butanol. A slow stream of air is bubbled through the mixture, usually for one minute, to obtain good mixing. After allowing the liquid phases to separate by standing, the upper *iso*butanol layer is removed by aspiration and discarded. The aqueous phase, which must be clear**, is washed with 3.0 ml of ether; the ether is removed and discarded. An aliquot (usually 1.0 ml) of the aqueous phase, is taken for determination of the radioactivity of the organically-bound phosphate. When the procedure is performed on a control reaction mixture to which the enzyme is added after trichloracetic acid, it is found that over 99% of the radioactivity (*i.e.*, of the orthophosphate) is removed. Such a control is included with every experimental run and the radioactivity of this blank sample is subtracted from that in the experimental ones to correct for the small amount of orthophosphate not removed. This should always be very small.

Assay 2. The rate of liberation of orthophosphate from ADP is used in this assay. For economy reasons the assay is carried out with concentrations of ADP, which although higher than those used in Assay 1, are insufficient to saturate the enzyme as far as the rate of liberation of phosphate is concerned. At saturation levels of ADP, the rate of liberation of phosphate is some three times faster than obtained in the standard assay. The reaction mixture contains the following components (in μmoles), Tris buffer, pH 8.1, 70–100; $MgCl_2$, 5; ADP, 10; and enzyme. Final volume, 1.0 ml. The amount of enzyme should be such that the reaction rate remains

* This is not an accurate calculation of the absolute amount of phosphate incorporated and is used only as a convenient expression of the activity of the enzyme under the conditions of Assay 1.

** Occasionally the aqueous phase may not be clear after one extraction with *iso*butanol; when this happens, the removal of orthophosphate is incomplete. A second extraction usually eliminates the remaining phosphate satisfactorily.

References p. 285.

essentially linear with time during the incubation; this amount is usually below 1.5 units, as determined by Assay 1. Usually, two samples are run for 5 and 15 minutes, respectively. The incubation is carried out with shaking at 30°. One unit of enzyme is defined as the amount which catalyzes the liberation of 1 μmole of orthophosphate per 15 min at 30°. The specific activity is defined as units per mg of protein. The reaction is stopped with trichloracetic acid as in Assay 1 and orthophosphate determined by the method of LOHMANN AND JENDRASSIK[13]. The rate of phosphate liberation is proportional to the concentration of enzyme between the limits of 0 and 1.5 (Assay 1) units. When the enzyme is free or almost free of adenylic kinase, the rate of phosphate liberation remains constant for at least 1 hour. In the presence of adenylic kinase it may be necessary to calculate the reaction rate from the 5 minute incubation value.

As shown in Fig. 3, the reaction rate, under the conditions of either Assay 1 or 2, is proportional to the concentration of enzyme over a fairly wide range of enzyme dilutions. It may also be seen that the ratio of phosphate incorporation (Assay 1) to that of phosphate liberation rate (Assay 2) is approximately 2.5–2.8. Assay 1 has been the one routinely used for purification of the enzyme. Unless otherwise stated, units and specific activities of the enzyme preparations throughout this paper are expressed in terms of Assay 1.

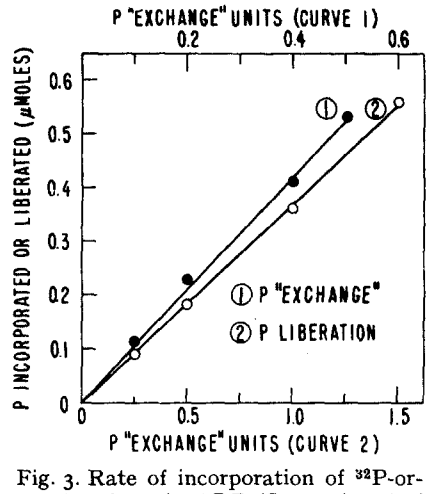

Fig. 3. Rate of incorporation of ^{32}P-orthophosphate in ADP (Curve 1) and of phosphate liberation from ADP (Curve 2) as a function of enzyme concentration. Curve 1 was obtained with a solution of enzyme (1.02 mg of protein per ml) of specific activity 10.6; Curve 2, with a solution of enzyme (1.08 mg of protein per ml) of specific activity 22.5. Specific activities are expressed in terms of assay 1. The ordinate represents μmoles of phosphate incorporated or liberated in 15 minutes at 30°.

Purification of enzyme

Azotobacter vinelandii (strain original)* was grown as described by HYNDMAN *et al.*[14]. The cells were harvested after 15 hours of growth; at this time a 1:5 dilution of the medium should read about 115 in a Klett colorimeter with filter No. 540. The yield was around 6 g of wet cells per liter of medium.

Step 1. Extraction. The enzyme, which is easily soluble, can be extracted from fresh, acetone-dried, or lyophilized cells. Fresh or dried cell preparations retain their activity for a long time when stored at —18°.

The fresh cells are ground in a mortar with double their weight of alumina A301 (325 mesh, Aluminum Company of America) at 0°, then extracted with 4.5 volumes of 0.15 *M* KCl and centrifuged for 30 minutes at 3000–4500 *g*. The residue of cells and alumina is re-extracted with 2.0 volumes of 0.15 *M* KCl for 30 min and the mixture is centrifuged as before. The supernatant extracts are combined and centrifuged for 30 minutes at 0° and 16,000 r.p.m. in the high speed head of the International Centrifuge. The supernatant fluid is then dialyzed against 0.01 *M* phosphate buffer, pH 7.0, for 2 hours with stirring. In the case of dried cell preparations, the extraction is carried out in the same way, except that an equal weight of alumina and 22.5 and 10.0 volumes, respectively, of 0.15 *M* KCl are used. The extract contains from 8–15 mg of protein per ml. An amount of extract containing 0.5–1.0 mg of protein is used per assay (Assay 1). The specific activity of the extract varies from 0.3 to 0.5.

Step 2. First ammonium sulfate fractionation. The extract is diluted, if necessary, with 0.01 *M* phosphate buffer, pH 7.0, to contain 10 mg of protein per ml and cooled to 0°. Solid ammonium sulfate is added with mechanical stirring to make 0.35 saturation. After stirring for 5 minutes, the mixture is centrifuged in the cold room at top speed in the Servall angle centrifuge (20,000 *g*). The precipitate, which contains β-hydroxybutyric dehydrogenase, has been used for the purification of this enzyme; this preparation will be described elsewhere. Solid ammonium sulfate is added to the supernatant solution as above to make its degree of saturation 0.46. The precipitate contains most of the polynucleotide phosphorylase activity. This fraction, which contains from 20 to 25% of the protein of the extract, is dissolved in 0.01 *M* potassium phosphate buffer, pH 7.4, and dialyzed with stirring in the cold for 2 hours against the same buffer. An amount containing about 0.2 mg of protein is used per assay. The specific activity of this fraction is about 2.0.

Step 3. Adsorption and elution from calcium phosphate gel. The dialyzed solution of the 0.35–0.46 ammonium sulfate precipitate is diluted, if necessary, with 0.01 *M* potassium phosphate

* We are indebted to Dr. P. W. WILSON, Department of Bacteriology, School of Agriculture, University of Wisconsin, Madison, Wisconsin, for a culture of the organism.

References p. 285.

buffer, pH 7.4, to contain 9–10 mg of protein per ml and cooled to 0°. To 95 ml of the enzyme solution is added 0.9 ml of 1.0 *M* acetate buffer, pH 5.1. The final pH of this solution is 5.8 and the salt concentration about 0.02 *M*. 15 ml of calcium phosphate gel[15] (dry weight, 20 mg per ml) are added to 96 ml of the above solution. After stirring for 20 minutes at 0° the gel, which contains about 9% of the protein of the fraction, and 5% of the activity, is centrifuged off and discarded. 5.0 ml of calcium phosphate gel are now added to the supernatant solution and the mixture is stirred and centrifuged as above. The gel is eluted first with 30–40 ml of 0.1 *M* potassium phosphate buffer, pH 6.0, at 0° and, after centrifugation in the cold, with 15 ml of 0.01 *M* potassium phosphate buffer, pH 7.3. The buffers used for elution are at 0° but the elutions are carried out at room temperature. The eluates are combined and dialyzed at 0° against 0.01 *M* potassium phosphate buffer pH 7.4, for 2 hours with stirring. An amount of eluate containing about 0.05 mg of protein is used per assay. The specific activity of the eluate, which contains about 18% of the protein of the fraction with 76% of the activity, is 8.0 to 10.0. The overall recovery of enzyme in the supernatant fluid and gel eluates is about 90%.

Step 4. Second ammonium sulfate fractionation. The dialyzed eluate, containing about 2.5 mg of protein per ml, is fractionated at 0° with saturated ammonium sulfate adjusted to pH 7.4 with ammonium hydroxide. Three fractions are obtained: fraction 1, between the limits of 0 and 0.45 ammonium sulfate saturation, fraction 2 between 0.45 and 0.55 saturation, and fraction 3 between 0.55 and 0.60 saturation. The ammonium sulfate precipitates are collected by centrifugation in the cold room (about 5°) at high speed in the Servall Angle centrifuge, dissolved in a small volume of 0.01 *M* potassium phosphate buffer, pH 7.4, and dialyzed at 0° with stirring against the same buffer for several hours. Fraction 1, which contains about 27% of the protein and 15% of the activity, and fraction 3 containing 10% of the protein and 11% of the activity of the eluate, are discarded. Fraction 2, which contains 22% of the protein and 73% of the activity of the eluate is the best preparation of the enzyme so far obtained. An amount of fraction containing from 0.02 to 0.03 mg of protein is used per assay; its specific activity is from 18 to 22. The overall recovery of activity in the three ammonium sulfate fractions amounts to 99%.

A summary of the purification procedure, starting with acetone-dried cells, is given in Table III. This run was particularly successful both as regards purification and yield. In two large-scale preparations from fresh cells, carried out subsequently*, the specific activity of the enzyme at stage 4 was 14.6 and 21.6, with a yield of 28 and 24%, respectively. The ammonium sulfate steps are generally reproducible but the degree of success with the calcium phosphate gel step depends on the particular gel preparation or batch used and the procedure finally utilized at this stage may have to deviate from the one described here.

TABLE III

PURIFICATION OF POLYNUCLEOTIDE PHOSPHORYLASE OF *Azotobacter vinelandii*

Step	*Volume ml*	*Units (Assay 1)*	*Protein mg*	*Ratio* 280/260*	*Specific activity units/mg protein*	*Yield per cent*
1. Dialyzed extract	376	1840	3450	0.5	0.5	100
2. $(NH_4)_2SO_4$ (0.35–0.46)	95	1750	810		2.2	95
3. $Ca_3(PO_4)_2$ gel eluate	58	1500	142	0.7	10.6	82
4. $(NH_4)_2SO_4$ (0.45–0.55)	5	1020	45	1.1	22.5	56

15.0 g of acetone-dried cells (*A. vinelandii*, strain original) extracted with an equal weight of alumina and 400 ml of 0.15 *M* KCl.

* Ratio of light absorption at λ280 to that at λ260 mμ.

In the run of Table III, besides Assay 1, on which the specific activities given in the table are based, Assay 2 was also used at each step. The specific activities based on this assay at steps 1, 2, 3, and 4, respectively, were 0.17, 0.95, 3.30, and 8.30 (*cf.* Table III). Thus, the ratios of rate of phosphate incorporation to rate of phosphate liberation, *i.e.*, the ratios of specific activities determined by Assay 1 to those determined by Assay 2, at each step, were 2.9, 2.3, 3.2, and 2.7, for steps 1 through 4, respectively, the average value of the ratio being 2.76 (*cf.* also Fig. 3). Thus, throughout the purification so far achieved of the *Azotobacter* enzyme, the ratio of the rate of phosphate incorporation to that of phosphate liberation remained constant.

* By Dr. M. STAEHELIN and Mr. M. C. SCHNEIDER.

References p. 285.

Properties of enzyme

Stability. Preparations of the enzyme at any step of purification remain active for periods as long as one year if kept in the frozen state at —18°. However, the enzyme loses activity on thawing, especially on repeated freezing and thawing. Although the enzyme is relatively stable at an acid pH (4.5 to 5.5), it cannot be stored frozen at this pH and loses about 70% of its activity on thawing under these conditions. Heating for 5 minutes at 50° and pH 5.8 destroys 50% of the enzyme activity. However, only 10 to 20% is lost if this heating is carried out at pH 6.4. The enzyme is completely destroyed by heating for 5 minutes at 60° and pH 5.6, or for 1 minute at 100° and pH 7.4.

pH Optimum. Under the conditions of Assay 1 the phosphate incorporation reaction has a rather sharp optimum at pH 8.1. At pH 6.4 the activity is 28% and at pH 10.0, 50% of that at pH 8.1. Under the conditions of Assay 2, the phosphate liberation reaction has a plateau of maximum activity between pH 7.5 and pH 9.0; at pH 6.4 the activity is 30% lower.

Affinity constants. Under the conditions of Assay 1 the system is saturated with concentrations of ADP of the order of $2.5 \cdot 10^{-3} M$ and concentrations of $MgCl_2$ between 1 and $2 \cdot 10^{-3} M$. The corresponding half saturation concentrations were $2.5 \cdot 10^{-4} M$ and $5 \cdot 10^{-4} M$, respectively. Much higher concentrations of nucleoside diphosphates are required to saturate the system in the case of Assay 2. Thus, saturating concentrations of ADP and IDP were of the order of $10^{-1} M$ and $5 \cdot 10^{-2} M$, respectively. The half saturating concentrations were $2.7 \cdot 10^{-2} M$ and $10^{-2} M$, respectively. The concentrations of $MgCl_2$ required to saturate the system of Assay 2 were about the same as those in Assay 1, namely between 1 and $2 \cdot 10^{-3} M$; the half saturation concentration for $MgCl_2$ was $4 \cdot 10^{-4} M$. Whereas small variations in the ratio of the concentration of nucleoside diphosphate to that of orthophosphate do not appear to influence significantly the reaction rate in the case of Assay 2, in that of Assay 1 the rate of incorporation of orthophosphate is markedly dependent on the above concentration ratio. In this case, the optimum concentration ratio of nucleoside diphosphate to orthophosphate was 0.7 for ADP and 1.0 for IDP. The reaction rates decrease rather sharply for values of the concentration ratio above or below the optimum.

TABLE IV

EFFECT OF INHIBITORS

Addition to assay system		*Per cent inhibition*	
Test substance	*Concentration (molar)*	*Assay 1*	*Assay 2*
Potassium arsenate	0.004	7	0
Potassium arsenate	0.01	44	0
Potassium fluoride	0.04	13	0
p-chloromercuribenzoate	0.00015	27	18
2,4-dinitrophenol	0.0005	0	
AMP	0.01	10	0
ATP	0.002	10	
Sodium pyrophosphate	0.01	18	

Enzyme of specific activity 16 with 0.037 mg of protein (0.6 unit) used for the first five experiments. Enzyme of specific activity 12 with 0.048 mg of protein (0.58 unit) used for the last three experiments. Conditions of standard assays.

References p. 285.

Inhibitors. As shown in Table IV, the enzyme is relatively insensitive to poisons being essentially unaffected by fluoride and by low concentrations of arsenate. Higher concentrations of the latter (0.01 *M*) inhibited 44% the incorporation reaction, although they had no effect on the liberation of orthophosphate. *p*-Chloromercuribenzoate had relatively little effect; hence, SH groups do not seem to be essential for the activity of the enzyme. The incorporation reaction (Assay 1) was relatively little affected by the presence of AMP, ATP or pyrophosphate. On the whole this reaction was more sensitive to inhibitors than the liberation of orthophosphate.

Synthesis of polynucleotides

The incorporation of orthophosphate into nucleoside diphosphates and the liberation of phosphate therefrom occur not only with the diphosphates so far considered, namely ADP and IDP, but also with any of the other diphosphates such as GDP, UDP, and CDP, or with mixtures of two or more nucleoside diphosphates. Table V illustrates the incorporation of ^{32}P-orthophosphate, measured as described for Assay 1,

TABLE V

^{32}P "EXCHANGE" WITH NUCLEOSIDE DIPHOSPHATES SINGLY AND MIXED

Nucleoside diphosphate μmoles	*^{32}P incorporated*	
	Expt. 1 c.p.m.	*Expt. 2 c.p.m.*
ADP, 2.0	16,600	108,000
GDP, 1.0	8,000	29,000
UDP, 4.0	22,500	142,300
CDP, 2.0	10,200	79,600
ADP + GDP + UDP + CDP*	1,270	5,800
ADP + GDP + UDP + CDP**		13,600

The samples contained the following components (in μmoles per ml), Tris buffer, pH 8.0, 100; $MgCl_2$, 5; potassium phosphate (pH 8.0) with 95,000 c.p.m. ^{32}P in experiment 1 and 690,000 c.p.m. ^{32}P in experiment 2, 2; enzyme of specific activity 18 with 0.063 mg of protein (1.12 units) in experiment 1 and of specific activity 14 with 0.046 mg of protein (0.7 unit) in experiment 2; and nucleoside diphosphates as indicated. Other conditions as in standard assay 1.

* Each in same amount as when tried singly.

** Each in half the amount as when tried singly.

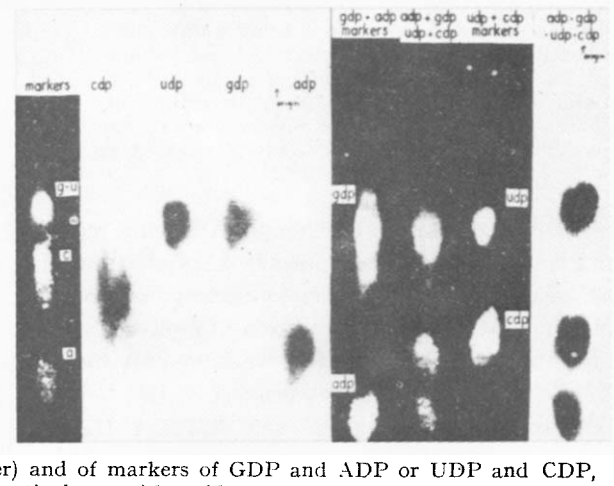

Fig. 4. Enzymic incorporation of ^{32}P-orthophosphate in nucleoside diphosphates singly and in combination. Conditions as in phosphate incorporation experiments of Table V. Paper chromatography by the method of KREBS AND HEMS[8]. (A) Strip to the left, U.V. print of markers of GDP (G), UDP (U), CDP (C), and ADP (A). GDP and UDP are not separated by the *iso*butyric acid-ammonia solvent system; strip to the right, autoradiograms of separate experiments with single nucleoside diphosphates. (B) Strip to the right, autoradiogram of experiment with simultaneous incubation of all four nucleoside diphosphates; strip to the left, U.V. print of simultaneous incubation experiment (center) and of markers of GDP and ADP or UDP and CDP, respectively, on either side.

References p. 285.

in the presence of ADP, GDP, UDP, and CDP, or a mixture of the four nucleoside diphosphates. The autoradiograms of Fig. 4 show that the radioactive phosphate is incorporated into each of the diphosphates whether incubated singly or in combination. These results suggested that the enzyme is able to catalyze the synthesis of either "single" polynucleotides, *i.e.*, polynucleotides containing a single basic unit, or "mixed" polynucleotides, *i.e.*, polynucleotides containing two or more basic nucleotide units. As already reported[2] this was confirmed by subsequent work.

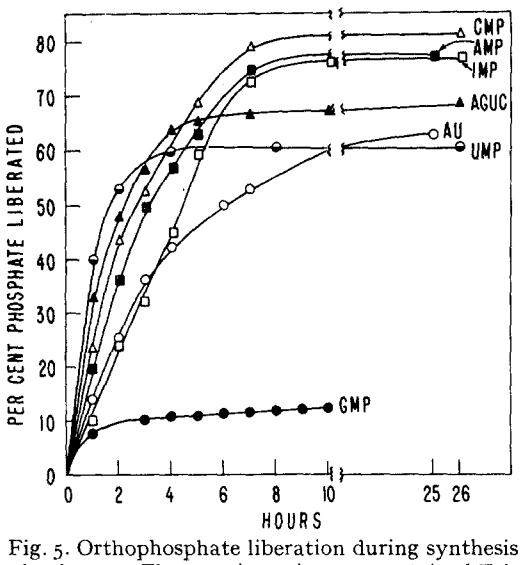

Fig. 5. Orthophosphate liberation during synthesis of polymers. The reaction mixtures contained Tris buffer, pH 8.1, 50, and $MgCl_2$, 10 μmoles per ml. For "single" polymers 50 mg per ml were used of each of the following nucleoside diphosphates, ADP, IDP, UDP and CDP, and 25 mg per ml of GDP. The sample for A–U polymer contained 25 mg of each ADP and UDP per ml; that for A–G–U–C polymer contained 7 mg per ml of each ADP, UDP and CDP, and 3.5 mg per ml of GDP. The samples for GMP and A–G–U–C polymers contained, per ml, 2.5 mg of enzyme of specific activity 9.5 (24 units); all others contained 0.5 mg of the same enzyme (4.75 units). A drop of toluene was added to each sample as a bacteriostat. Final volume, 0.5 ml. Incubation with shaking at 30°. 0.02 ml aliquots were removed as indicated for orthophosphate determination. The ordinate gives the percentage of acid-labile phosphate released as orthophosphate.

On incubation of purified polynucleotide phosphorylase with nucleoside diphosphates singly or in combination, in the presence of Mg^{++}, there is a liberation of orthophosphate. The reaction reaches equilibrium and comes to a standstill when 60 to 80% of the acid labile phosphate has been released as orthophosphate. The time course of the reaction with ADP, IDP, GDP, UDP and CDP, or with mixtures of either ADP and UDP, or ADP, GDP, UDP, and CDP, is shown in Fig. 5. Addition of more nucleoside diphosphate when the liberation of orthophosphate has stopped disturbs the equilibrium and causes further liberation of phosphate until a new equilibrium position is reached. The polynucleotides which accumulate in the reaction mixtures are usually isolated by precipitation with cold ethanol and purified by solution in a small volume of water, reprecipitation with ethanol, solution, and exhaustive dialysis against distilled water. They are then recovered from the dialyzed solution by lyophilization, and obtained in the form of white powders.

It may be seen in Fig. 5 that all nucleoside diphosphates except GDP react readily. In the case of GDP the reaction is much slower and often comes to a standstill when a small fraction of the diphosphate has reacted. A precipitate, containing both polynucleotide and protein, is frequently obtained at this point. Addition of more enzyme brings about further reaction which, however, soon stops. So far, we have only been able to obtain small amounts of the GMP polynucleotide. It is because of this behavior that GDP is used at lower concentration than other nucleoside diphosphates and that much more enzyme is utilized for the preparation of polymers containing GMP.

Preparation and isolation of polymers. Single polymers have been prepared from

References p. 285.

ADP, IDP, GDP, UDP, or CDP, and are referred to as the AMP, IMP, GMP, UMP, or CMP polymers, respectively. To date, two "mixed" polymers have been prepared, one from approximately equimolar mixtures of ADP and UDP and one from mixtures of ADP, GDP, UDP, and CDP, in approximate molar proportion, 1:0.5:1:1; these polymers are referred to as the A-U, and the A-G-U-C polymers, respectively. The composition of the samples per ml of the reaction mixture is, in general, as indicated in the legend to Fig. 5. Since the incubation periods are of 24 hours or longer, a small amount of toluene is added to the reaction mixtures to prevent bacterial growth.

The polymers are isolated as follows:

Any precipitate which may be present in the samples at the end of the incubation is removed by centrifugation at low temperature and low speed. The clear solution is then cooled to 0°, two volumes of ice-cold absolute ethanol are added, and the mixture is allowed to stand at 0° for one hour. The precipitated polymer is centrifuged off for 10 minutes at 12,000 *g* and 0°; the supernatant fluid is decanted and the precipitate is dissolved in a minimum amount of distilled water. The polymer is reprecipitated once with two volumes of ice-cold absolute ethanol and the precipitate collected and dissolved in water as above. Any insoluble material at this point is removed by centrifugation and discarded. The clear, colorless polymer solution is dialyzed against several changes of distilled water in the cold room (about 5°) for 48 hours and the dialyzed solution dried from the frozen state.

Detailed information on individual preparations, including composition of the reaction mixtures, the extent to which the reaction proceeded as judged from the liberation of orthophosphate, the yield of lyophilized material and the percentage recovery as polynucleotide of the diphosphates disappearing, are recorded in Table VI.

It will be noted that in order to avoid degradation of the polymers by heat or acid, protein is not removed prior to the precipitation of the polynucleotides with alcohol. Protein determinations in the solutions of polymers after the second alcohol precipitation indicated that about 70% of the protein had been removed. Thus, the

TABLE VI

DATA ON SYNTHESIS OF VARIOUS POLYMERS

No.	Reaction mixture: Nucleoside diphosphate mg	Tris buffer pH 8.1 μmoles	$MgCl_2$ μmoles	Enzyme units	Total vol. ml	Extent of reaction* per cent	Yield of lyophilized material mg	Recovery** as polynucleotide per cent
1	ADP, 1000	1000	200	80	20	60	356	82
2	UDP, 450	450	90	40	9	40	75	65
3	UDP, 200	200	40	20	4	59	43	56
4	ADP, 180 UDP***, 180	360	72	32	7	46	88	87
5	ADP, 100 UDP***, 100	200	40	19	4	44	62	90–100
6	IDP, 200	200	40	19	4	80	82	80
7	CDP, 150	150	30	16	3	70	40	63
8	ADP, 200 GDP, 100 UDP, 200 CDP, 200	1400	280	725	28	64	154	64

Incubation with shaking at 30°, 40 to 46 hours.

* As determined from orthophosphate liberation.

** Calculated for composition of nucleoside diphosphates as follows: ADP, $NaH_2ADP \cdot 2H_2O$ GDP, $Na_3GDP \cdot 3H_2O$; UDP, $Na_3UDP \cdot 3H_2O$; CDP, $Na_3CDP \cdot 4H_2O$; IDP, $Na_3IDP \cdot 4.4H_2O$.

*** Correction was made for an inorganic impurity present in this UDP sample.

References p. 285.

amount of protein remaining and contaminating the polymer preparations is insignificant, except in the case of the AGUC polymer in which the amount of enzyme used was much larger than in the others.

Phosphorolysis

The reaction catalyzed by polynucleotide phosphorylase is reversible. In the presence of the enzyme, orthophosphate and Mg^{++}, the biosynthetic polynucleotides undergo phosphorolysis to yield the corresponding 5'-nucleoside diphosphates. There is no reaction in the absence of orthophosphate. Quantitative data on the stoichiometry of the reaction with IDP in both directions have been presented previously[1]. We have as yet no accurate data on the position of the equilibrium. In the direction of polynucleotide synthesis and under our usual experimental conditions (pH 8.1, 30°), the reaction comes to a standstill when the ratio of the concentration of orthophosphate to that of nucleoside diphosphate is from 1.5 to 2. This is the case with ADP or IDP. Under these conditions the reaction favors polynucleotide synthesis, as would be expected from the fact that the pyrophosphate bonds of nucleoside diphosphates are converted to the phosphodiester bonds of the polynucleotide.

TABLE VII

PHOSPHOROLYSIS OF BIOSYNTHETIC POLYMERS AND NUCLEIC ACIDS

Experiment No.	*Polymer*	*Source of polymer*	*^{32}P added c.p.m. · 10^{-6}*	*Enzyme units**	*^{32}P incorporated c.p.m.*	*Orthophosphate disappearance μmoles*
1	AMP	Synthetic	3.65	9.3	1,380,000	2.75
	RNA	*A. vinelandii*			16,000	0
	AMP + RNA				320,000	1.85
2	AMP	Synthetic	1.68	9.3	860,000	1.50
	RNA	*A. vinelandii*			25,600	0
	RNA + IDPase				11,600	0
	AMP + RNA				276,000	0.40
3	AMP	Synthetic	0.31	4.6	71,000	0.80
	UMP	Synthetic			85,000	0.70
	A–U	Synthetic			28,000	
4	IMP	Synthetic	0.35	6.2	82,000	0.58
	RNA	*A. vinelandii*			7,600	
	RNA followed by IDPase				3,850	
	IMP + RNA				60,500	0.50
5	AMP	Synthetic	0.55	6.2	200,000	1.10
	RNA	*E. coli*			11,700	0
	AMP + RNA				100,000	
	RNA	*Str. pyogenes*			11,700	0
6	AMP	Synthetic	0.82	4.6	380,000	
	RNA	Yeast			18,800	
	RNA	Ox liver			9,300	
	RNA	Wheat germ			5,800	

Samples contained (in μmoles per ml), Tris buffer, pH 8.1 in experiments 1 to 3, pH 7.0 in experiments 4 to 6, 100; $MgCl_2$, 5; potassium phosphate, 7 to 9 with ^{32}P as indicated; polymer, 0.5 to 3.0 mg; and enzyme (specific activity 15 to 20) as indicated. Final volume, 1.5 ml. Incubation 90 minutes at 30°. When two different polymers were present, equal amounts of each were added. Values expressed per ml of reaction mixture.

* Assay 1.

References p. 285.

Phosphorolysis of "single" polymers can be readily detected and measured by the disappearance of orthophosphate. The biosynthetic A-U and A-G-U-C polymers are also phosphorolyzed although less readily than "single" polymers. Moreover, the enzyme can catalyze the phosphorolysis of natural RNA from *Azotobacter vinelandii* as well as from other sources. However, with the exception of turnip yellow mosaic virus RNA*, nucleic acids, including that from *Azotobacter*, do not seem to be phosphorolyzed easily. Under these conditions one may be unable to detect disappearance of orthophosphate. Nevertheless, the occurrence of phosphorolysis can be readily detected by the use of ^{32}P-orthophosphate which is incorporated into the nucleoside diphosphates, and these can be identified chromatographically and autoradiographically. Calf thymus DNA and a sample of yeast RNA "core" *i.e.*, the fraction of RNA which following exhaustive digestion with pancreatic ribonuclease is non-dialyzable against distilled water, were not attacked[2]. Thus, as far as phosphorolysis is concerned, the *Azotobacter* polynucleotide phosphorylase does not appear to be specific for *Azobacter* RNA.

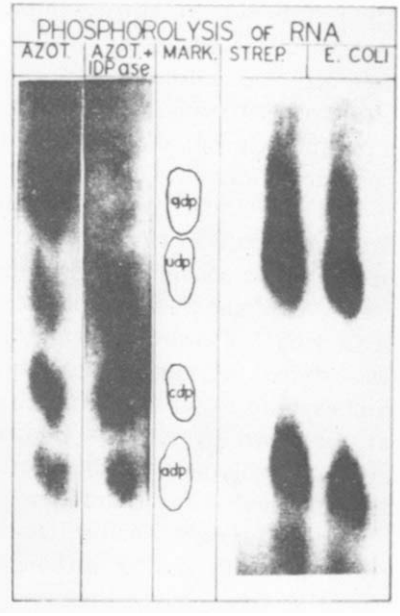

Fig. 6. Phosphorolysis of RNA. Samples of RNA from *Azotobacter vinelandii* (first two autoradiograms from left), *Streptococcus pyogenes* and *Escherichia coli* (last two autoradiograms) were used. Aliquots of the corresponding samples of experiments 4 and 5 of Table VII, following deproteinization, were chromatographed on paper by the method of KREBS AND HEMS[8] and autoradiograms obtained. The center strip is the sketch of a chromatographic strip with markers (from top to bottom) of GDP, UDP, CDP and ADP. The second strip from left was derived from a sample incubated with IDPase (see text) before chromatography.

Table VII shows data on the phosphorolysis of biosynthetic polymers and natural ribonucleic acids by the *Azotobacter* enzyme. Both the incorporation of labeled phosphate (determined as described for Assay 1) and the disappearance of orthophosphate were measured in most cases. Only in the case of biosynthetic polynucleotides was there a measurable disappearance of orthophosphate although incorporation of labeled phosphate occurred throughout. It may further be seen (Experiments 1, 2, 4 and 5) that RNA inhibited to a greater or lesser extent the phosphorolysis of the AMP or the IMP polymer. Attention is called to the RNA phosphorolysis of Experiments 2 and 4 in which a separate aliquot contained, or was subsequently incubated with, inosine diphosphatase (IDPase). This enzyme[16] catalyzes the hydrolysis of IDP, GDP and UDP to the corresponding 5'-nucleoside monophosphates but is inactive on ADP and CDP. Where IDPase was used, the radioactivity incorporated was halved indicating that the enzyme hydrolyzed the GDP and UDP (produced by phosphorolysis) with release of the terminal radioactive phosphate as orthophosphate. This conclusion is supported by the autoradiograms of Fig. 6. Comparison with the markers shown on the central strip suggests that the two upper spots on the first strip correspond to GDP and UDP, whereas the two lowest spots on this strip correspond to CDP

* L. A. HEPPEL and J. D. SMITH, unpublished experiments.

References p. 285.

and ADP. The middle spot is probably labeled ATP formed from labeled ADP by adenylic kinase contaminating the *Azotobacter* enzyme preparation. It may be seen (second strip from left) that the two upper spots largely disappeared from the sample treated with IDPase following incubation with RNA. The last two strips in Fig. 5 suggest the formation of radioactive GDP, UDP, CDP and ADP by phosphorolysis of RNA from *Escherichia coli* or *Streptococcus pyogenes*. They correspond to two of the samples of Experiment 5 in Table VII.

DISCUSSION

The results of work already published[1,2] and of further work to be reported in detail in subsequent papers of this series, show that the polynucleotides synthesized by polynucleotide phosphorylase consist of 5'-nucleoside monophosphate units linked to one another through 5'-3'-phosphoribose diester bonds. The chains are terminated by a phosphate group esterified at carbon 5' of the nucleoside moiety*. The biosynthetic polynucleotides of Table VI have average chain lengths ranging from 30 for the A-G-U-C polymer to 230 for the AMP polymer*. Ultracentrifugal studies by Dr. R. C. WARNER in this laboratory suggest average molecular weights ranging from 70,000 to 350,000 for these polynucleotides. Higher values, namely 570,000 and 800,000 for an AMP and an IMP polymer, respectively, were obtained by the method of light scattering**. However, during the preparation of the latter polymers the equilibrium was shifted at various time intervals by supplementary additions of nucleoside diphosphate to promote further synthesis. It is possible that polymers of larger size are obtained by multiple additions of nucleoside diphosphates. Some of the biosynthetic polymers can be stretched into fibres[17] from which Dr. ALEXANDER RICH has obtained X-ray diffraction patterns suggesting a high degree of orientation***. "Single" polymers, such as the AMP polynucleotide, give X-ray diffraction patterns which are very similar, although not identical to those given by natural RNA. On the other hand, biosynthetic polynucleotides containing both purine and pyrimidine bases, such as the A-U and the A-G-U-C polymer, give patterns virtually identical to those of natural RNA. Thus, the information at present available on structure, base ratios, size, X-ray diffraction patterns, and behavior toward different enzymes[2] indicates that the "mixed" polynucleotides synthesized by polynucleotide phosphorylase from 5'-nucleoside diphosphates are closely related to RNA. Indeed, the A-G-U-C polymer, *i.e.*, the one containing adenylic, guanylic, uridylic and cytidylic acid residues, appears to be indistinguishable from natural RNA.

It cannot as yet be decided whether the reaction catalyzed by polynucleotide phosphorylase represents a general biological mechanism for the synthesis of ribonucleic acids. Wide distribution of the enzyme would favor such a possibility. Recent work§ indicates that the distribution of the enzyme is rather wide. It has been found in extracts of a number of bacterial cells, irrespective of whether the organisms are aerobic or anaerobic, gram positive or gram negative. The enzyme has also been found in yeast extracts and in extracts of green plants (spinach). However, no unequivocal evidence has yet been obtained for the presence of the enzyme in extracts

* J. D. SMITH and L. A. HEPPEL, unpublished end-group assays.
** L. F. CAVALIERI and M. ROSOFF, unpublished experiments.
*** A. RICH, unpublished experiments.
§ D. O. BRUMMOND, M. STAEHELIN and M. GRUNBERG-MANAGO, unpublished experiments.

References p. 285.

of animal tissues. It may be that polynucleotide phosphorylase is present in all cells although in widely different amounts. Larger amounts would be expected in cells which have high rates of proliferation.

Polynucleotide phosphorylase may prove useful for gaining further knowledge of the chemistry and the physico-chemical properties of nucleic acids as well as in studies on the mechanism of action and specificity of nucleases. Since the enzyme can catalyze the synthesis of polynucleotide chains containing either a single mononucleotide or two or more different nucleotides (some of which may not occur naturally in nucleic acids), a number of non-naturally occurring polynucleotides can be obtained for studies of various kinds. It has been found*, for example, that "single" polynucleotides containing only adenylic or guanylic acid rapidly interact in aqueous solution to form a new polymer of much higher molecular weight than that of each of the initial reactants. This polymer migrates as a homogeneous compound in an electric field.

Little is as yet known of the mechanism of action of polynucleotide phosphorylase. Studies aimed at elucidating this point may have to await further purification of the enzyme. The question of whether the enzyme, in analogy with muscle phosphorylase, can only add nucleotide units to a pre-existing polynucleotide chain, *i.e.*, whether the enzyme requires a primer, or whether it can build a polynucleotide chain starting only from a mixture of nucleoside diphosphates is of obvious interest. No definite answer can yet be given to this question. In unpublished experiments, Dr. L. A. HEPPEL has so far failed to find evidence for accumulation of small polynucleotides on brief incubation of the enzyme with nucleoside diphosphates. The possibility that a nucleoside monophosphate, for example adenosine-5′ monophosphate, reacts initially with a nucleoside diphosphate, such as uridine-5′-diphosphate, to give a dinucleotide has been eliminated by Dr. HEPPEL by end-group assays of the polynucleotide synthesized.

Azotobacter polynucleotide phosphorylase shows considerable lack of specificity since it can act on single nucleoside diphosphates, whether naturally or non-naturally occurring, or on mixtures of two or more of these. It also lacks specificity in the direction of phosphorolysis as it can attack ribonucleic acids from various sources. However the possibility that, in the presence of adequate concentrations of the four naturally occurring nucleoside diphosphates, the enzyme could synthesize a polynucleotide chain with a determined sequence of nucleotides, *i.e.*, a specific RNA, is indeed intriguing. It is conceivable that RNA bound on the enzyme might act as a prosthetic group and a template for the reproduction of its own nucleotide pattern. Answers to this question might be obtained through studies now under way of the polynucleotides synthesized by phosphorylases from different sources.

Finally, an interesting corollary of the reversibility of the reaction catalyzed by polynucleotide phosphorylase is that the free energy of hydrolysis of the phosphodiester bonds in polynucleotides may not be much lower than that of the pyrophosphate bonds of nucleoside diphosphates.

METHODS AND PREPARATIONS

The analytical methods used have been described or referred to in the experimental sections. For the determinations of radioactivity, aliquots of deproteinized reaction mixtures, before and

* R. C. WARNER, unpublished experiments.

References p. 285.

after removal of orthophosphate, were counted directly as liquid samples with use of a thin-window-Geiger-Müller counter. Since the radioactivity of the orthophosphate was determined in every experiment under exactly the same conditions as that of other experimental samples, no corrections were applied for self-absorption or decay.

Azotobacter RNA was prepared from the residue (particles) obtained by high speed centrifugation of the bacterial extract during the first step of isolation of polynucleotide phosphorylase. The material was kept frozen until used. 100 g of residue were extracted in a Waring blendor for 15 minutes at 3–4° with 10 volumes of 0.14 *M* sodium chloride. The mixture was centrifuged at 3–4° at high speed in a Servall angle centrifuge (20,000 *g*). The sediment was re-extracted as above with 2 volumes of 0.14 *M* sodium chloride and the extracts were combined. The combined extracts were heated for 1 hour at 85° and the protein precipitate was removed by centrifugation after cooling. To the supernatant fluid was added 40% trichloroacetic acid to give a final concentration of 5%. The precipitate was collected by centrifugation, stirred in 0.1 *M* Tris buffer, pH 7.35, and the insoluble residue centrifuged off and discarded. The supernatant fluid was treated once more with trichloroacetic acid as above. The precipitated nucleic acid was dissolved in Tris buffer, pH 7.35, leaving no insoluble residue, and the solution was dialyzed with stirring against distilled water at 0° for 48 hours. Based on U.V. absorption at wave-length 260 mμ, the yield of nucleic acid at this point was 39.0 mg. The solution was adjusted to pH 7.0 and the nucleic acid was precipitated with 3 volumes of ethanol. The precipitate was collected by centrifugation, washed twice with ethanol and once with ether, and dried in vacuum over P_2O_5. An aqueous solution of the powder after dialysis contained 26% DNA as determined by the DISCHE reaction[18]. Before use for the experiments of Table VII, the solution of nucleic acid was incubated for 1 hour at 30° with an excess of crystalline deoxyribonuclease, in the presence of $MgCl_2$, and dialyzed against water. The DNA content was reduced to 18%.

ATP and ADP were obtained commercially from the Sigma Chemical Co. and the Pabst Laboratories. The crystalline disodium salt of ATP was obtained from the Sigma Chemical Laboratories.

We are deeply indebted to Mr DAN BROIDA, Sigma Chemical Co., St. Louis, Mo., for generous gifts of large amounts of UDP, GDP, IDP and other nucleotides. We are also much indebted to Dr. ALEXANDER FRIEDEN and Dr. SAMUEL A. MORELL, Pabst Laboratories, Milwaukee, Wisc., for generous gifts of UDP and CDP. The present work would not have been possible without the above help. Our thanks are due to Dr. A. W. BERNHEIMER, Dept. of Microbiology, New York University College of Medicine, for samples of bacterial, wheat germ and ox liver RNA, to Dr. J. R. FRESCO Dept. of Pharmacology, New York University College of Medicine, for help with the preparation of *Azotobacter* RNA and for samples of yeast RNA and calf thymus DNA, and to Dr. L. A. HEPPEL, National Institutes of Health, Bethesda, Md., for a sample of yeast RNA "core". IDPase was kindly supplied by Dr. G. W. E. PLAUT. We are indebted to Dr. M. KUNITZ Rockefeller Institute for Medical Research, New York, N.Y., for a gift of crystalline yeast hexokinase.

SUMMARY

The isolation, partial purification and some properties of polynucleotide phosphorylase of *Azotobacter vinelandii* are described. The enzyme catalyzes the synthesis of highly polymerized ribonucleic acid-like polynucleotides from 5′-nucleoside diphosphates with release of orthophosphate. The reaction requires magnesium ions and is reversible. Thus, the enzyme also catalyzes the phosphorolysis of polynucleotides to yield the corresponding 5′-nucleoside diphosphates. The preparation and isolation of a number of polynucleotides containing one or several kinds of mononucleotide units is described. The scope, mechanism and significance of the reaction are discussed.

References p. 285.

REFERENCES

1 M. GRUNBERG-MANAGO AND S. OCHOA, *J. Am. Chem. Soc.*, 77 (1955) 3165.
2 M. GRUNBERG-MANAGO, P. J. ORTIZ AND S. OCHOA, *Science*, 122 (1955) 907.
3 H. SCHMITZ, R. B. HURLBERT AND V. R. POTTER, *J. Biol. Chem.*, 209 (1954) 41.
4 V. R. POTTER, *Canadian Cancer Conference* (R. W. BEGG, editor), Vol. I, New York, 1955, p. 290.
5 M. GRUNBERG-MANAGO AND S. OCHOA, *Federation Proc.*, 14 (1955) 221.
6 H. M. KALCKAR, *J. Biol. Chem.*, 148 (1943) 127.
7 W. E. COHN AND C. E. CARTER, *J. Am. Chem. Soc.*, 72 (1950) 4273.
8 H. A. KREBS AND R. HEMS, *Biochem. Biophys. Acta*, 12 (1953) 172.
9 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
10 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
11 I. A. ROSE AND S. OCHOA, *J. Biol. Chem.*, (in press).
12 I. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
13 K. LOHMANN AND L. JENDRASSIK, *Biochem. Z.*, 178 (1926) 419.
14 L. A. HYNDMAN, R. H. BURRIS AND P. W. WILSON, *J. Bacteriol.*, 65 (1953) 522.
15 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. (London), B*, 124 (1938) 397.
16 G. W. E. PLAUT, *J. Biol. Chem.*, 217 (1955) 235.
17 A. RICH AND J. D. WATSON, *Proc. Natl. Acad. Sci. U.S.*, 40 (1954) 759.
18 Z. DISCHE, *Mikrochemie*, 8 (1930) 4.

Received December 30th, 1955