*POLYMERIZATION OF NUCLEOSIDE DIPHOSPHATE WITH A MANGANESE-DEPENDENT ENZYME FROM ESCHERICHIA COLI Q_{13}**

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From the extensive research carried out on various organisms it is apparent that there are several different forms of polynucleotide phosphorylase. This differentiation has been made on the basis of the ability of the enzyme to catalyze the synthesis or degradation of polynucleotides,[1-5] the heat lability[3] and molecular weight of the enzyme,[1-3] the requirement for a sulfhydryl reagent such as β-mercaptoethanol,[2] and the effect of polyamines on the rate of the reaction.[2, 4, 5] It has also been shown that Mg^{++}, the divalent cation standardly used in most assay systems of polynucleotide phosphorylase, may be replaced by manganous ions and to a varying extent by other divalent cations.[6] Polymerization of guanosine diphosphate to polyguanylate occurs with either cation present if the temperature of the incubation is raised to 60°C.[7] The strict requirement for oligonucleotide primer observed in some instances[8] may be a result of the method of isolation of the enzyme.[9]

This report describes the isolation of a Mn-dependent form of polynucleotide phosphorylase from *E. coli* Q_{13}, a mutant which has been reported to be defective in RNase I and polynucleotide phosphorylase.[10-12]

Experimental Procedure.—Materials: Unlabeled nucleotides were obtained from P-L Biochemicals, Inc.; C^{14}-ADP, C^{14}-GDP, C^{14}-UDP, and C^{14}-CDP from Schwarz Biochemical Co.; α-P^{32}-ADP from International Chemical and Nuclear Corps.; P^{32}-phosphoric acid from Tracer Labs.; poly A, poly C, and poly U from Miles Chemical Co.; pancreatic DNase and RNase from Worthington Biochemical Co.; creatine phosphate and creatine phosphokinase from Boehringer Mannheim Corp.; polylysine from Sigma Chemical Co.

Assays: The standard assay for this enzyme, modified from assay A of Littauer and Kornberg,[13] measures the conversion of C^{14}-labeled ribonucleoside diphosphate into acid-insoluble form. The incubation mixture (0.1 ml) consisted of 100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 10 mM $MnCl_2$, 5 mM C^{14}-ADP (0.1 μc/μmole). The reaction was initiated by the addition of enzyme solution. After incubation of the vessels for 10 min at 37°C, the reaction was terminated by the addition of 0.02 ml of 5% bovine serum albumin and 3 ml of 5% trichloroacetic acid (TCA). The precipitate was collected on glass fiber filter paper (Carl Schleicher and Schuell Co. No. 29) and was washed with 5% TCA. The washed glass fiber filter paper was placed in the scintillation vial which contained 10 ml of Bray's scintillation solution.[14] The radioactivity was counted with a Nuclear-Chicago scintillation counter. One unit of enzyme was defined as the amount catalyzing the conversion of 1 mμmole C^{14}-ADP into acid-insoluble form in 10 min. The amount of protein was determined by the procedure of Warburg and Christian.[15]

For details of the assays used for the phosphorolysis of polynucleotides and for the measurement of the exchange of P^{32}-orthophosphate with nucleoside diphosphate, see the legends of Figures 7 and 8, respectively. The method for the hydrolysis of P^{32}-labeled polynucleotides and the separation of the nucleotides is reported in the legend of Table 2.

Results.—Purification of enzyme: The enzyme was extracted from *E. coli* Q_{13} (Hfr, methionine$^-$, tyrosine$^-$, RNase I$^-$, polynucleotide phosphorylase$^-$), which was provided by Dr. W. Gilbert. *E. coli* Q_{13} was grown in a medium which consisted of 1 per cent NaCl, 1 per cent Difco Bacto-tryptone, 0.5 per cent Difco Bacto-Yeast Extract, and 0.25 per cent glucose. All operations of fractionation

were performed at 0–4°C. The centrifugations were performed at 20,000 × g for 30 minutes.

(1) *Preparation of crude extract:* Frozen cells (5 gm) and 10 gm of levigated alumina (Alundum, Norton Co., Worcester, Mass.) were vigorously ground for 10 minutes. Thirty ml of 0.01 M Tris-HCl, pH 7.8, were added to the ground paste. The mixture was stirred for 20 minutes and centrifuged. The clear supernatant fluid was collected by decantation and was diluted with the same buffer until the absorbancy at 260 mμ was 100.

(2) *Streptomycin sulfate–pH 5–ammonium sulfate fractionation:* To 35 ml of this extract were added 3.5 ml of 5 per cent streptomycin sulfate solution with stirring. After 15 minutes, the suspension was centrifuged and the supernatant solution was collected. The supernatant fluid was adjusted to pH 5 with 0.5 M acetic acid. After 15 minutes, the suspension was centrifuged and the precipitate was discarded. To 36 ml of supernatant fluid were slowly added 14.4 gm of ammonium sulfate. After 15 minutes the suspension was centrifuged and the supernatant fluid discarded. The precipitate was suspended by use of a glass homogenizer in a solution prepared by dissolving 3 gm of ammonium sulfate in 20 ml of 0.01 M Tris-HCl, pH 7.8. The homogenate was centrifuged and the precipitate was discarded. To the supernatant fluid were slowly added 5 gm of ammonium sulfate. After 15 minutes the suspension was centrifuged. The precipitate was dissolved in 5 ml of 0.01 M Tris-HCl (pH 7.3)–0.005 M $MgCl_2$. The solution was dialyzed against 1 liter of this same buffer solution overnight with two changes of buffer solution.

(3) *DEAE-cellulose column chromatography:* The dialyzed solution was applied to the DEAE-cellulose column at a rate of 30 ml per hour. DEAE-cellulose was washed according to Peterson and Sober[16] and equilibrated in 0.01 M Tris-HCl (pH 7.3)–0.005 M $MgCl_2$. The column of 1 cm in diameter and 12 cm in length was packed under 5 psi. After application of the enzyme solution, the column was washed with 20 ml of 0.01 M Tris-HCl (pH 7.3)–0.005 M $MgCl_2$, 20 ml of 0.1 M Tris-HCl (pH 7.3)–0.005 M $MgCl_2$, and 50 ml of 0.2 M Tris-HCl (pH 7.3)–0.005 M $MgCl_2$, successively. The enzyme was eluted from the column with 0.4 M Tris-HCl (pH 7.5)–0.005 M $MgCl_2$. The active fractions were combined. To each milliliter of combined solution was slowly added 0.4 gm of ammonium sulfate. After 15 minutes, the suspension was centrifuged. The precipitate was dissolved in 0.05 M Tris-HCl (pH 7.8) and dialyzed against 1 liter of the same buffer overnight with two changes of buffer solution. The dialyzed enzyme solution was divided into 0.2-ml portions and stored at −15°C. The enzyme is stable for at least two weeks at this temperature.

The purification procedure and the results of a typical preparation are summarized in Table 1. All experiments were performed with the most highly purified enzyme.

TABLE 1

PURIFICATION OF THE NUCLEOSIDE DIPHOSPHATE POLYMERIZING ENZYME FROM *E. coli* Q_{13}

Fraction	Volume (ml)	$\frac{OD_{280}}{OD_{260}}$	Spec. act. (units/mg protein)	Total act units
Extract	35	0.55	36	12,000
Streptomycin sulfate–pH 5–ammonium sulfate	8	1.05	210	8,400
DEAE-cellulose–ammonium sulfate	1.6	1.45	638	4,080

Properties of the enzyme: (1) *Metal ion requirements:* Manganous ion was required for the polymerization of adenosine diphosphate and could not be replaced with an equivalent amount of magnesium ion. As shown in Figure 1 the rate of the reaction at pH 8.0 increased in proportion to the concentration of manganous ion up to 10 mM. At this concentration of metal ion and at the standard concentration of ADP (5 mM) the assay solution became slightly cloudy, probably as a result of the precipitation of the manganous salt of ADP. For this reason standard assays were not made beyond a concentration of 10 mM Mn^{++} ion and 5 mM nucleoside diphosphate.

(2) *The effect of pH and concentration of adenosine diphosphate:* The maximal rate of reaction was reached at pH 8.0 and 2.5 mM ADP (Figs. 2 and 3). Above this pH and ADP concentration the reaction rate remained constant and was not affected by the turbidity which appeared in some vessels where the solubility limits of some components were exceeded.

(3) *The utilization of nucleoside diphosphates for polymer formation:* As in Figure 4, corresponding homopolymers may be formed from CDP and UDP in addition to ADP. Under the conditions of our assay at 37°C, however, GDP is not converted significantly into polyguanylate. When all four labeled nucleoside diphosphates were added to the assay system at a concentration of 1.25 mM each, the rate of polynucleotide synthesis was considerably below that for the reaction of ADP, UDP, or CDP alone. As will be shown below, the product formed from all four nucleoside diphosphates is a heteropolymer containing the purine and pyrimidine bases in approximately equal amounts.

Such a complete analysis has not been made on polymers formed from two C^{14}-labeled substrates (added at a concentration of 2.5 mM each). However, it seems likely that they are also heteropolymers. The rate of formation of these mixed polynucleotides as compared to the rate of synthesis of the three homopolymers is shown in the following decreasing order: poly CU, poly C, poly AC, poly A, poly AU, poly AG, poly U, and poly UG (Fig. 4). Poly GC was not formed. GDP therefore seems to have a pronounced effect on the utilization of CDP.

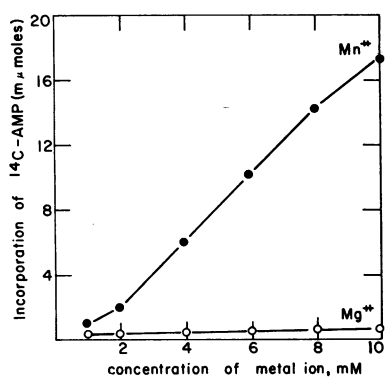

FIG. 1.—The influence of concentration and nature of metal ions on the rate of polymerization of C^{14}-ADP. Standard assay conditions were used, except that the metal ion concentration was varied as shown: ●—● Mn^{++} alone added to the assay system, ○—○ Mg^{++} alone added to the assay system.

The nature and nucleotide composition of the product formed from α-P^{32}-ADP under two different experimental conditions were examined. In the first, α-P^{32}-ADP (3,000 cpm/mμmole) was the only nucleoside diphosphate (5 mM) included in the incubation system; in the second, the composition of the nucleoside diphosphate was 1.25 mM each of the unlabeled UDP, CDP, GDP, and α-P^{32}-ADP (3,000 cpm/mμmole). The total concentration of nucleoside phosphates in the two vessels thus remained the same, i.e., 5 mM.

Polymerized product was subjected to alkaline degradation and electrophoretic separation of the mononucleotides. The dis-

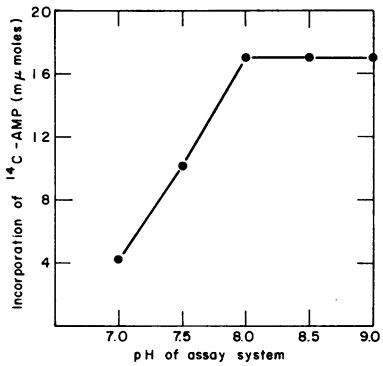

FIG. 2.—The effect of pH on the rate of polymerization of C^{14}-ADP. Standard assay systems were used, except that the pH of 0.1 *M* Tris-HCl was varied as shown.

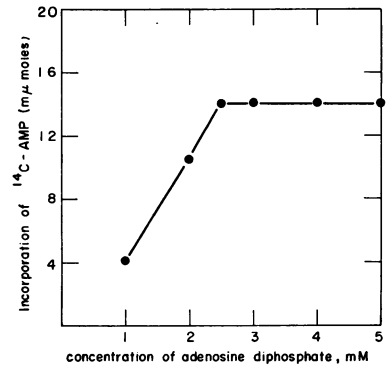

FIG. 3.—The effect of concentration of adenosine diphosphate on the rate of polymerization of C^{14}-ADP. Standard assay system was used, except that the concentration of adenosine diphosphate was varied as shown.

tribution of P^{32} among isolated nucleotides was, therefore, a measure of the composition of the newly synthesized RNA. The results shown in Table 2 indicated that in the first system ADP was polymerized into acid-insoluble but alkali-sensitive product, which contains solely AMP units. In the second system, the product after hydrolysis yielded equally P^{32}-labeled 3′ (or 2′) AMP, UMP, CMP, and GMP, an indication that the heteropolymer poly ACUG was produced when all four nucleoside diphosphates were available simultaneously to the enzyme. Again, in confirmation of experiments with C^{14}-labeled nucleoside diphosphates, the rate of synthesis of poly ACUG as measured by the incorporation of α-P^{32}-ADP was considerably slower than the rate of poly A formation even after taking into account that the concentration of α-P^{32}-ADP in the second vessel was only one fourth that of the first and that ADP competed with the other three nucleoside diphosphates for incorporation into the polymer.

(4) *The effect of DNase, RNase, and phosphorylating systems on the formation of polynucleotides:* DNase had no effect on the rate of the conversion of the three nucleoside diphosphates into their respective homopolymers. As would be ex-

TABLE 2

ANALYSIS OF HOMO- AND HETEROPOLYMERS FORMED FROM α-P^{32}-ADP FOR THE DISTRIBUTION OF P^{32} IN THE NUCLEOTIDES FORMED BY ALKALINE HYDROLYSIS

Reaction system containing	Radioactivity in 3′ (2′) nucleotides (cpm)			
	CMP	AMP	CMP	UMP
(1) α-P^{32}- ADP	—	45,700	—	—
(2) α-P^{32}-ADP with CDP, UDP, and GDP	760	800	750	850

The reaction system (0.5 ml) consisted of 100 mM Tris-HCl, pH 8.0, 10 mM $MnCl_2$, 0.25 *M* EDTA, and 5 mM α-P^{32}-ADP (3,000 cpm/mμmole) or 1.25 mM each of UDP, CDP, GDP, and α-P^{32}-ADP (3,000 cpm/mμmole). The purified enzyme was added to initiate the reaction. After 60 min at 37°C, 0.1 ml of 5% bovine serum albumin and 3 ml of 5% perchloric acid were added to the system. The suspension was centrifuged. The precipitate was washed twice with 3 ml of 5% perchloric acid. The washed precipitate was dissolved in 0.2 ml of 0.3 *N* KOH. The solution was incubated at 38°C for 18 hr. The incubated solution was neutralized to pH 3 with 70% perchloric acid. The suspension was centrifuged. The supernatant fluid was mixed with 0.02 ml of a solution containing 100 mμmoles each of the four nucleoside 3′ (2′) monophosphates and applied to Whatman paper 3 MM. The paper was subjected to electrophoresis at pH 3.5 in 0.05 citrate buffer at 2 kv for 3 hr. The individual nucleotides, which were located with a UV lamp, were cut out and placed in the scintillation vial. The scintillation solution was added to fill up the vial and the radioactivity of each nucleotide was counted.

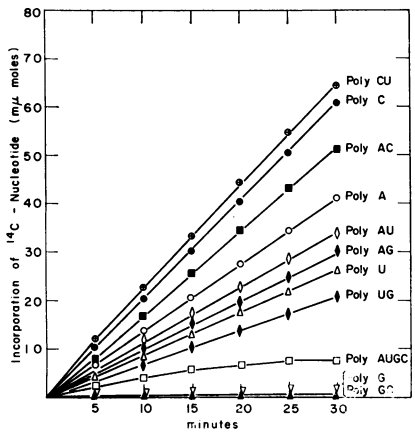

FIG. 4.—The rate of formation of polynucleotides. The standard assay systems were used, except that C^{14}-ADP, C^{14}-ADP, C^{14}-CDP, and C^{14}-GDP were varied as shown. For copolymers two C^{14}-nucleoside diphosphates were used at a concentration of 2.5 mM each. The reaction mixture for poly AUGC synthesis contained 1.25 mM each of the C^{14}-nucleoside diphosphates.

pected from previous experience with other forms of polynucleotide phosphorylase, the reaction catalyzed by the enzyme from *E. coli* Q_{13} is not DNA-directed.

RNase completely inhibited the formation of the homopolymers from the pyrimidine nucleoside diphosphates, UDP and CDP. Since RNase does not split diester bonds of homopolymers of the purine nucleotides, the polymerization of ADP to poly A was not affected by this degradative enzyme.

At this stage of purification of the enzyme, approximately 18-fold, we have detected no requirement for any oligonucleotide primer.

The polymerization of all three nucleoside diphosphates into their respective homopolymers is inhibited by inclusion of a phosphorylation system in the reaction mixture. Both the creatine phosphate–creatine phosphokinase and the phosphoenol pyruvate–pyruvic phosphokinase systems were equally effective. These experiments demonstrate that the nucleoside diphosphates are the actual substrates of the polymerization and that upon phosphorylation to the triphosphate level, they are no longer available for enzymatic reaction. In confirmation of this experiment it was found that the nucleoside triphosphates did not serve as substrates. Likewise, C^{14}-labeled dCDP either alone or in the presence of dTDP, dADP, and dGDP was not converted enzymatically into a C^{14}-acid-insoluble product. The enzyme thus catalyzes specifically the polymerization of ribonucleoside diphosphates.

(5) *The effect of polylysine on the formation of polynucleotides:* The formation of poly A was stimulated more than twofold by addition of polylysine (Fig. 5). Manganous ions are essential for the stimulation of the reaction by polylysine and cannot be replaced by magnesium ions. β-Mercaptoethanol is without effect on the reaction either in the presence or absence of polylysine.

In contrast, the formation of poly C from CDP was strongly inhibited by polylysine and the synthesis of poly U, poly G, and poly AUGC was not affected by the polypeptide (Fig. 6).

A precipitate, which was observed in the assay system containing both ADP and polylysine, was removed by centrifugation before the reaction was initiated. There was, however, no appreciable loss of soluble, ultraviolet-absorbing substances resulting from this operation.

(6) *Phosphoroylsis of polynucleotides:* Under all conditions tested, the rate of phosphoroylsis of polynucleotides was extremely slow or negligible (Fig. 7). In an attempt to find circumstances under which phosphorolysis would take place, the conditions of the assay were systematically varied: (*a*) the pH was changed from

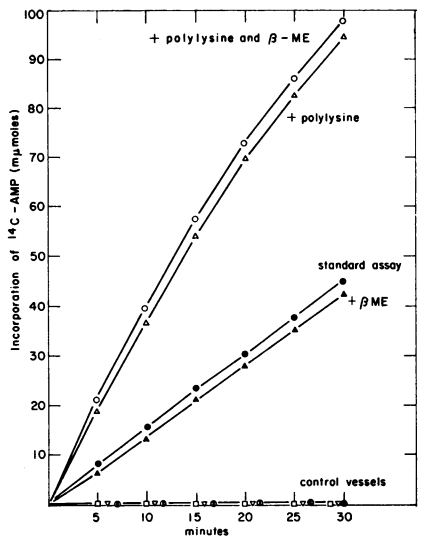

FIG. 5.—The effect of polylysine on the rate of polymerization of C^{14}-ADP. When indicated, polylysine (mol. wt 2,600 or 190,000 at a concentration of 280 μg/ml and/or β mercaptoethanol (β-ME) at a final concentration of 0.001 *M* were added to the standard assay system. ● Standard assay; ▲ plus β-ME; ○ plus polylysine and β-ME; △ plus polylysine; □ standard assay but Mg^{++} replaced for Mn^{++}; ▽ standard system but Mg^{++}, polylysine, and β-ME replaced for Mn^{++}; ① standard assay but without enzyme.

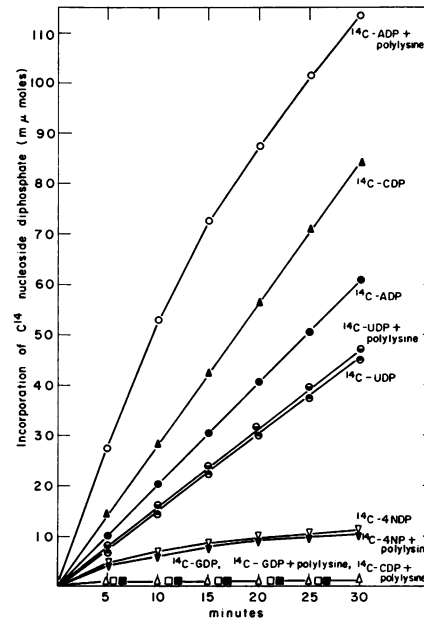

FIG. 6.—The effect of polylysine on the rate of polymerization of C^{14}-ADP, C^{14}-CDP, C^{14}-UDP, and C^{14}-GDP into homo- and heteropolymers. The standard assay system was used except that the nucleoside diphosphates C^{14}-ADP, C^{14}-GDP, C^{14}-UDP, C^{14}-CDP were varied as shown. When added alone the concentration of each of the nucleoside diphosphates was 5 mM. When all four were added together the concentration of each was 1.25 mM. Where indicated, polylysine (mol. wt. 2,600) was added at a concentration of 280 μg/ml.

8 to 7.5 and 8.5; (*b*) the nature and concentration of the divalent cation was changed from 0.0025 *M* $MgCl_2$ to 0.01 *M* $MgCl_2$ and to 0.002 *M* $MnCl_2$; (*c*) poly A was replaced as the substrate by poly U, poly C, *E. coli* tRNA, *E. coli* ribosomal RNA, and yeast RNA. None of the changes had any effect on the amount of P^{32}-nucleoside diphosphate formed from polynucleotide (or RNA) and P^{32}-orthophosphate. We cannot understand at present the differences between the results of these experiments and those reported by Thang *et al.*[1]

(7) *Exchange reaction between P^{32}-orthophosphate and nucleoside diphosphate:* As with the phosphorolysis of polynucleotides reported above, we could find no conditions under which a significant exchange of P^{32}-orthophosphate and nucleoside diphosphate occurred (Fig. 8). The pH of the standard assay (for exchange) was changed from 8 to 7.5 and 8.5: the concentration and nature of the divalent cation was varied from 0.005 *M* $MgCl_2$ to 0.01 *M* $MgCl_2$ or 0.002 *M* $MnCl_2$, and ADP was replaced by GDP, UDP, or CDP as substrate.

Discussion and Summary.—E. coli Q_{13} reportedly possesses a very low and unstable polynucleotide phosphorylase activity,[10] an observation which has been interpreted as resulting from a lack of this enzyme in addition of RNase I.[11, 12] Using any one of the three conventional systems for the assay of polynucleotide

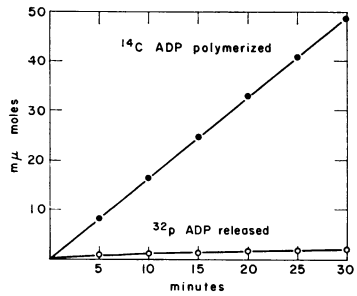

FIG. 7.—Studies on the phosphorolysis of polynucleotides. The standard assay for phosphorolysis, a modification of the method of Singer,[17] measures the conversion of poly A and P^{32}-orthophosphate into charcoal-adsorbable P^{32} form. The assay system (0.1 ml) consisted of 100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 2.8 mM (nucleotide) poly A, and 10 mM P^{32}-phosphate (~500 cpm/mμmole). The reaction was initiated by the addition of enzyme solution. After incubation for 10 min at 37°C, the reaction was terminated by the addition of 1 ml of charcoal suspension, which contained 1% charcoal and 2.5% perchloric acid. After incubation of suspension in ice water for 10 min, 3 ml of 1% perchloric acid and 1 mM KH_2PO_4 were added and the suspension was centrifuged. The precipitate was washed twice and suspended with 3 ml of the same solution. This extra washing was necessary to decrease the background of radioactivity of substrates absorbed to the charcoal. The charcoal was collected on the glass fiber filter paper and was washed with 10 ml of ice water three times. The washed glass filter fiber was placed on the planchet with the charcoal side downward. The radioactivity was counted in a Nuclear-Chicago gas-flow counter. One unit of enzyme was defined as the amount catalyzing the release of 1 mμmole of P^{32}-adenosine diphosphate in 10 min. The formation of P^{32}-ADP in the phosphorolysis assay is indicated by open symbols; the rate of polymerization of ADP by a comparable amount of enzyme under the standard assay conditions described in the experimental section is indicated by solid symbols.

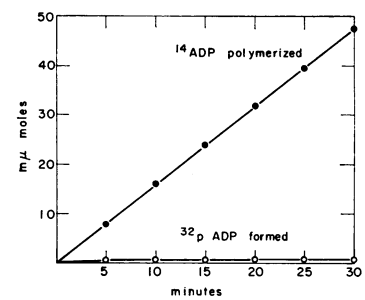

FIG. 8.—Studies on the exchange of P^{32}-orthophosphate and the β phosphate of adenosine diphosphate. The standard assay for exchange reaction is a modification of assay C of Littauer and Kornberg[13] and measures the conversion of adenosine diphosphate and P^{32}-phosphate into charcoal-adsorbable P^{32}-form. The assay system (0.1 ml) consisted of 100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 5 mM $MgCl_2$, 20 mM P^{32}-potassium phosphate (~500 cpm/mμmole), and 16 mM ADP. The reaction was initiated by the addition of enzyme solution. After an incubation period of 10 min at 37°C, the reaction was terminated by the addition to the vessels of 1 ml of charcoal suspension, which contained 1% charcoal and 2.5% perchloric acid. After incubation of the suspension in ice water for 10 min, the charcoal was washed and counted as described in the standard assay of phosphorolysis (see Fig. 7). One unit of enzyme was defined as the amount catalyzing the formation of 1 mμmole P^{32}-adenosine diphosphate in 10 min. The formation of P^{32}-ADP in the exchange assay is indicated by open symbols; the rate of polymerization of ADP by a comparable amount of enzyme under the standard assay conditions described in the experimental section is indicated by solid symbols.

phosphorylase, each of which employs Mg^{++} as the source of divalent cation, we have been able to confirm the very low activity of this enzyme in crude extracts of *E. coli* Q_{13}. However, Thang, Thang, and Grunberg-Manago[1] have reported the phosphorolysis of polynucleotides and the exchange reaction of inorganic phosphate with nucleoside diphosphate in enzyme preparations from *E. coli* Q_{13}. The polymerization reactivity of Q_{13} extracts, reported by them, however, is quite low.

In *E. coli* Q_{13} we have observed the existence of a form of polymerizing activity with the following catalytic properties: (1) The enzyme catalyzes the formation of high-molecular-weight homopolymers of adenosine, uridine, and cytidine monophosphate from the corresponding diphosphates. The rate of formation of the heteropolymer of adenosine, uridine, guanosine, and cytidine mono-

phosphate is relatively slow. (2) The phosphorolysis of polynucleotides and the exchange between inorganic phosphate and the terminal phosphate of nucleoside diphosphate proceeds at a negligible or extremely low rate. (3) The polymerization of nucleoside diphosphates requires the presence of Mn^{++} ion, and Mg^{++} ion seems to be without effect on the reaction. (4) Polylysine stimulates the synthesis of polyadenylate from ADP, inhibits the formation of polycytidylate, and is without effect on the formation of polyuridylate. (5) β-Mercaptoethanol has no effect on the catalytic activity of the enzyme.

We are not certain at this stage of the enzyme purification whether the requirement for manganous ion is a fundamental property of the enzyme or whether manganous ions inhibit side reactions that in some way dissipate products or reactants of the polynucleotide phosphorylase system. In contrast to the normal enzyme system from *E. coli* B in which manganous ions replace magnesium ions for all three reactions of polynucleotide phosphorylase,[6] manganous ions are effective only in the synthesis of polynucleotides in the *E. coli* Q_{13} system and do not promote phosphorolysis or the exchange reaction. Under the conditions of our assay systems magnesium ions fail to support any of the three assay reactions with the enzyme from *E. coli* Q_{13}. The enzyme isolated from *E. coli* Q_{13} seems to differ in certain essential properties from polynucleotide phosphorylase obtained from other strains of *E. coli* and from other microorganisms.

Abbreviations used are: DEAE-cellulose, diethylaminoethyl-cellulose; EDTA, ethylene diamine tetraacetate; β-ME, β-mercaptoethanol; Tris, tris (hydroxymethyl) aminomethane.

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