SOME ENZYME REACTIONS WITH ADENINE DEOXYRIBOSIDE POLYPHOSPHATES*

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INTRODUCTION

In the preceding communication[1] the isolation and identification of deoxy-ADP*** and deoxy-ATP from reaction mixtures of ATP, deoxy-AMP and myokinase were described. In the present paper some enzyme-catalyzed reactions with deoxy-ADP or deoxy-ATP as substrates are presented. It was found that these two substrates can participate as phosphate acceptors or phosphate donors, respectively, in some phosphate transfer reactions. Furthermore, deoxy-ATP was found to serve as deoxyadenyl donor in the presence of NMN and DPN-pyrophosphorylase, resulting in the formation of a deoxyribose analog of DPN.

MATERIALS AND METHODS

AMP, ADP, DPNH and the crystalline sodium salt of ATP were products of the Sigma Chemical Company, and deoxy-AMP was a product of California Foundation for Biochemical Research. Phosphopyruvic acid, DPN and crystalline glutamic acid dehydrogenase were products of Boehringer & Sohne, Mannheim, Germany. Deoxy-ADP and deoxy-ATP were prepared as previously described[1]. NMN was obtained from DPN by pyrophosphorolytic cleavage in the presence of excess pyrophosphate and DPN-pyrophosphorylase[2], which was prepared from yeast according to KORNBERG[2]. NMN was isolated as described by PLAUT AND PLAUT[3]. Myokinase and adenylic acid deaminase were prepared as described by COLOWICK and KALCKAR[4], crystalline pyruvic acid kinase as described by BÜCHER AND PFLEIDERER[5], lactic acid dehydrogenase according to KORNBERG[6], crystalline alcohol dehydrogenase according to RACKER[7], glucose-6-phosphate dehydrogenase according to LEPAGE AND MUELLER[8] and inorganic pyrophosphatase according to HEPPEL AND HILMOE[9]. DPN or DPN-like compounds were assayed spectrophotometrically at 340 mμ with alcohol dehydrogenase in the presence of 1.7 *M* ethyl alcohol and 0.04 *M* pyrophosphate buffer, pH 8.5. The molar extinction coefficient of $6.22 \cdot 10^3$ was used for reduced DPN[10]. Pyruvic acid kinase was assayed spectrophotometrically at 340 mμ in the presence of phosphopyruvate and DPNH and excess lactic acid dehydrogenase as described by BÜCHER AND PFLEIDERER[5], and myokinase in the presence of phosphopyruvate and DPNH and excess of pyruvic acid kinase and of lactic acid dehydrogenase as described by BEISENHERZ *et al.*[11], *i.e.* the decrease in absorption at 340 mμ was used as an indicator of the reactions. Orthophosphate was determined according to FISKE AND SUBBAROW[12].

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*** The following abbreviations are employed: Deoxy-AMP, deoxy-ADP and deoxy-ATP for deoxyadenosine mono-, di- and triphosphate; AMP, ADP and ATP for adenosine mono-, di- and triphosphate; DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide and NMN for nicotinamide mononucleotide.

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RESULTS

Pyruvic acid kinase

This enzyme preparation was found to catalyze the phosphorylation of deoxy-ADP by phosphopyruvic acid. The rate of the reaction was, however, only about 11% of that of the phosphorylation of ADP, when assayed under the same conditions (see Table I). Deoxy-ADP inhibited the rate of phosphorylation of ADP to some extent, when the two compounds were present in equimolar concentrations. The addition of catalytic amounts of ADP to the deoxy-ADP reaction mixture had little or no effect on the reaction rate, suggesting the absence of a possible nucleoside diphosphate kinase catalyzing the phosphorylation of deoxy-ADP by ATP. However, when a less fractionated pyruvate kinase was used, the presence of catalytic amounts of ADP increased the rate by about 100%.

TABLE I

PYRUVATE KINASE REACTIONS

Substrates (moles/liter)		*Reaction rates ($10^3 \times \Delta E_{340}$/min/γ protein)*	
ADP	*Deoxy-ADP*	*Cryst. pyruvate kinase*	*Less purified pyruvate kinase*
$2 \cdot 10^{-4}$	0	94	
0	$1.9 \cdot 10^{-4}$	10.5	4.0
$2 \cdot 10^{-4}$	$1.9 \cdot 10^{-4}$	87	
$5 \cdot 10^{-6}$	$1.9 \cdot 10^{-4}$	10.7	8.2

The reaction mixture (1.0 ml) contained: DPNH ($1.1 \cdot 10^{-4}$ *M*), phosphopyruvate ($2.4 \cdot 10^{-4}$ *M*), $MgCl_2$ (10^{-2} *M*), KCl ($8 \cdot 10^{-2}$ *M*), tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4, (0.2 *M*), lactic acid dehydrogenase and ADP, deoxy-ADP and pyruvate kinase as indicated in the table.

Myokinase-like reactions

In the preceding communication it was shown that myokinase preparations could catalyze the phosphorylation of deoxy-AMP by ATP and that two of the products of this reaction were deoxy-ADP and deoxy-ATP, and it was assumed that the following three reactions took place:

$$\text{Deoxy-AMP} + \text{ATP} \rightleftharpoons \text{ADP} + \text{deoxy-ADP} \quad (1)$$

$$2\ \text{deoxy-ADP} \rightleftharpoons \text{deoxy-AMP} + \text{deoxy-ATP} \quad (2)$$

$$2\ \text{ADP} \rightleftharpoons \text{AMP} + \text{ATP} \quad (3)$$

In order to obtain further evidence for these reactions the rates of the following four possible reactions between mono-and triphosphates:

$$\text{ATP} + \text{AMP} \rightleftharpoons 2\ \text{ADP} \quad (4)$$

$$\text{deoxy-ATP} + \text{AMP} \rightleftharpoons \text{deoxy-ADP} + \text{ADP} \quad (5)$$

$$\text{ATP} + \text{deoxy-AMP} \rightleftharpoons \text{ADP} + \text{deoxy-ADP} \quad (6)$$

$$\text{deoxy-ATP} + \text{deoxy-AMP} \rightleftharpoons 2\ \text{deoxy-ADP} \quad (7)$$

have been compared, both with myokinase and with crude dialyzed muscle extract. As assay the pyruvate kinase – lactic acid dehydrogenase system was used. Proportionality with rate of reaction was checked by using two different concentrations of

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myokinase or muscle extract. With crude muscle extract it was found that the rate of reaction (5) was around 60% of that of reaction (4), whereas the rate of reactions (6) and (7) was only about 10 and 5%, respectively, of that of reaction (4). About the same relative rates of the reactions were found with myokinase preparations, except that reactions (6) and (7) proceeded with somewhat slower relative rates than in the case of the crude extract.

DPN-pyrophosphorylase

KORNBERG[2] has shown that enzymes from liver or yeast catalyze the reversible synthesis of DPN and pyrophosphate from ATP and NMN. In the presence of inorganic pyrophosphatase the equilibrium of this reaction will shift completely towards DPN-synthesis, due to the irreversible hydrolysis of the pyrophosphate formed. The possibility that deoxy-ATP can participate in this reaction instead of ATP has been tested as follows. Aliquots of incubation mixtures of NMN, deoxy-ATP, DPN-pyrophosphorylase and excess inorganic pyrophosphatase were analyzed for orthophosphate and DPN-like compounds. It was found that increasing amounts of both of these compounds were formed with time. In Fig. 1 such a reaction is recorded, and for comparison also the lapse of the reaction between ATP and NMN under the same conditions is given. In both cases NMN was present in excess over deoxy-ATP or ATP, respectively. It is seen that in each of the experiments about two micromoles of phosphate (plotted as pyrophosphate) have been formed per micromole of triphosphate. The reaction rate was four to five times slower, when deoxy-ATP was used instead of ATP. It is further seen that in both cases DPN, or a DPN-like compound, is also formed during the reaction. The concentration of these compounds, however, is somewhat lower than the corresponding phosphate concentration (expressed as pyrophosphate), and in the case of DPN it is even decreasing at 160 minutes. This phenomenon was found to be due to a DPN-destroying enzyme activity (probably organic pyrophosphatase) present in the inorganic pyrophosphatase. Thus, when the amount of inorganic pyrophosphatase was only a third of that in the experiment mentioned above, the yield of DPN-analog at five hours of incubation was about 75% of the theoretically obtainable value. From an incubation mixture with these proportions of the components, and with the initial amount of 5 μmoles of deoxy-ATP, a DPN-analog has been isolated. After incubation for five hours at 30° C the reactions were stopped by heating the mixtures at 100° C for five minutes. This solution was passed through a column

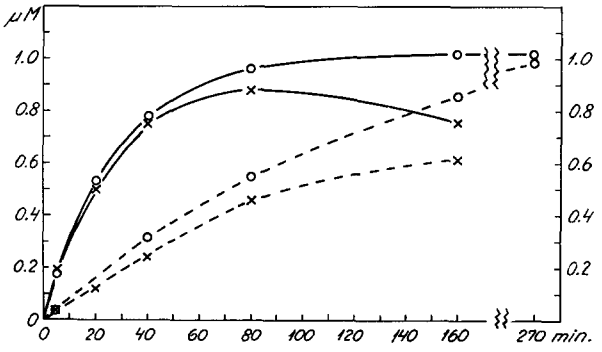

Fig. 1. Reaction between NMN and ATP or deoxy-ATP. The reaction mixtures (1 ml) contained: NMN ($1.25 \cdot 10^{-3}$ *M*), $MgCl_2$ ($2.5 \cdot 10^{-3}$ *M*), inorganic pyrophosphatase (0.2 ml), DPN-pyrophosphorylase (0.2 ml), glycylglycine buffer, pH 7.4, (0.05 *M*) and ATP (10^{-3} *M*) or deoxy-ATP (10^{-3} *M*). At time intervals aliquots were analyzed for orthophosphate and for DPN or DPN-like compounds. o = μmoles of orthophosphate, divided by 2, × = μmoles of DPN or DPN-like compounds, —— reaction mixture contained ATP and – – – reaction mixture contained deoxy-ATP.

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of Dowex-1 formate (0.8 cm^2 × 15 cm); the column was washed with about 100 ml of water and gradient elution was then carried out with ammonium formate, pH 5. The major ultraviolet-absorbing peak in the chromatogram had a $D_{280}:D_{260}$ ratio of about 0.33. The fractions containing this peak were pooled and treated with Norit[3] to remove the salts. The analysis showed a ratio of deoxyribose: ribose: total phosphate of 1:0.95:2.15. It is assumed therefore that the compound is a dinucleotide (deoxy-DPN) of NMN and deoxy-AMP. The yield of this compound was about 50% of the amount, which theoretically could be formed from the original amount of deoxy-ATP.

The deoxy-DPN was tested as hydrogen acceptor in some dehydrogenase systems. Thus with alcohol and alcohol dehydrogenase, deoxy-DPN (7.6×10^{-5} M) gave rise to light absorption at 340 mμ, suggesting that the compound acts as hydrogen acceptor. The rate of this process was, however, 50–60 times slower, than when DPN ($7.6 \cdot 10^{-5}$ M) was used as hydrogen acceptor under the same experimental conditions. The total change in optical density at 340 mμ corresponded to the reduction of 88% of the deoxy-DPN added, assuming the molar extinction coefficient of the reduced compound to be 6.22. The addition of deoxy-DPN to the alcohol dehydrogenase system containing DPN had little or no effect on the rate of reaction. With glutamic acid (10^{-2} M) and glutamic acid dehydrogenase in phosphate buffer, pH 7.7, the presence of deoxy-DPN ($7.6 \cdot 10^{-5} M$) gave rise to an increase in optical density with a rate, which was about a tenth of that obtained in the presence of DPN ($7.6 \cdot 10^{-5}$ M) under the same experimental conditions. It is therefore concluded that deoxy-DPN can serve as hydrogen acceptor in these dehydrogenase reactions. With glucose-6-phosphate ($5 \cdot 10^{-4}$ M) and glucose-6-phosphate dehydrogenase no increase in optical density was observed at 340 mμ in the presence of deoxy-DPN (10^{-4} M).

DISCUSSION

The finding that deoxy-ADP can serve as phosphate acceptor in the phosphopyruvate – pyruvate kinase system suggests, either that this enzyme catalyzes the reaction between deoxy-ADP and phosphopyruvate, or that a nucleoside diphosphate kinase ("Nudiki") is present in the enzymes used, and that this "Nudiki" catalyzes a phosphate transfer between deoxy-ADP and a contaminating amount of ATP. The ADP formed in this reaction would then be rephosphorylated by phosphopyruvate. The experiments with addition of catalytic amounts of ADP to the deoxy-ADP-phosphopyruvate reaction mixture indicate, however, that while crystalline pyruvate kinase and lactic acid dehydrogenase are free of a nucleoside diphosphate kinase catalyzing phosphate transfer between adenosine phosphate and deoxy-adenosine phosphates, such an enzyme is present in less purified preparations of pyruvate kinase. The possibility of the presence of other nucleoside diphosphate kinase systems in the reaction mixture used is, however, not excluded.

It is very likely that nucleoside diphosphate kinases are present in myokinase assay systems. However, the basis for the assay of the reactions (4) (5) (6) and (7) is the phosphate transfer from phosphopyruvate to either ADP or deoxy-ADP, either of which would be formed during the reactions. Since it has been shown that both of these diphosphates react in the phosphopyruvate – pyruvate kinase systems, the presence of a "Nudiki" would probably have no effect on the rate of formation or

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subsequent phosphorylation of the diphosphates, as long as the pyruvate kinase reactions are not rate-limiting.

In the case of reactions (5) and (6), both a triphosphate and a monophosphate different from those originally present in the incubation mixture, will be generated from the pyruvate kinase reaction. Myokinase-like reactions between the generated triphosphate and either of the two monophosphates and between the generated monophosphate and the original triphosphate will take place, therefore, to an increasing extent as the reactions proceed. The figures given for reaction rates were obtained over the first 3 to 5 minutes of the incubation. The time course of the reaction in this period was a straight line. Further fractionation of myokinase is needed before any definite conclusions can be drawn about the number of enzymes that catalyze the reactions (4) – (7).

It is of interest that not only reactions involving phosphate transfer, but also the DPN-pyrophosphorylase reaction, which can be regarded as a mononucleotide transfer reaction, can proceed (although more slowly) with deoxy-ATP as substrate instead of ATP. Examples of other nucleoside polyphosphates taking part in this reaction have not been reported. The possibility of chemical synthesis of deoxy-DPN and other DPN-analogs from NMN and another mononucleotide has been revealed by the finding of SHUSTER *et al.*[13] of the effect of trifluoro-acetic acid anhydride on mononucleotides.

The findings reported here suggest that for some of the enzymes catalyzing reactions with ATP as substrate the presence of the hydroxyl group at carbon No. 2 of the ribose is not absolutely necessary. It is interesting to speculate on the possibility of the existence of enzymes catalyzing reactions specific for deoxy-ADP or deoxy-ATP. The discovery of GRUNBERG-MANAGO *et al.*[14] of polynucleotide phosphorylase in some bacteria has disclosed the possibility of a similar formation of deoxyribopolynucleotide from deoxyribonucleoside polyphosphates.

Added in proof:

It has very recently been shown by KORNBERG *et al.*[15] that enzymes from *E. coli* catalyze the utilization of the polyphosphates of thymidine and of adenine, guanine and cytosine deoxynucleosides for DNA synthesis.

SUMMARY

It has been found that crystalline pyruvate kinase catalyzes the transfer of phosphate from phosphopyruvate to deoxy-ADP. The rate of this reaction is about 11 % of that of the reaction between phosphopyruvate and ADP. Evidence for the presence in muscle extract of a nucleoside diphosphate kinase, which catalyzes the transfer of phosphate from ATP to deoxy-ADP, has been obtained. This enzyme is not present in crystalline pyruvate kinase.

Crude muscle extract and preparations of myokinase contain enzymes catalyzing the transfer of phosphate from ATP or deoxy-ATP to AMP or deoxy-AMP. The relative rates of the reactions are given.

Deoxy-ATP was found to react with NMN in the presence of DPN-pyrophosphorylase and inorganic pyrophosphatase. The products of the reaction were found to be orthophosphate and a compound, the analysis of which suggests it to be a dinucleotide between deoxy-AMP and NMN. This compound gave rise to absorption at 340 mμ in the presence of either ethyl alcohol and alcohol dehydrogenase or glutamic acid and glutamic acid dehydrogenase.

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THE REACTION OF SILK FIBROIN WITH OXIDIZING AGENTS

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The general effect of oxidizing agents on proteins is to oxidize side-chains and bring about main-chain degradation. During an investigation to determine the latter by studying the viscosity of solutions of oxidized silk fibroin, it was found that many agents rendered much of the protein insoluble in the usual solvents.

Although it is known that fibroin may be made insoluble by cross-linking agents such as formaldehyde[14]; 1:3-difluoro-4:6-dinitrobenzene[19] and bis-(4-fluoro-3-nitrophenyl)sulphone[21], only in a review by HOWITT[8] is it stated that fibroin may be rendered insoluble in cupriethylenediamine by light irradiation, or reaction with chemical reagents. No experimental details are given.

In this paper results are presented which show that fibroin may be insolubilized by reaction with simple oxidizing agents such as chlorine, potassium permanganate, chlorine dioxide and iodine. It is shown also that the solubility of fibroin is highly dependent on the state of the tyrosine residues, and oxidation or substitution in the benzene nucleus of this side-chain may render fibroin insoluble.

EXPERIMENTAL

Raw silk (*Bombyx mori*) was freed from sericin and dirt by washing in warm soap solution, several changes of warm distilled water, and then standing in distilled water overnight. After removal of foreign matter by teasing, the dried silk was extracted with ethyl alcohol and then ether in a Soxhlet apparatus.

Oxidation reactions

The general procedure was to react 1 g of the purified fibroin with the reagent dissolved in 200 ml

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