A polynucleotide coenzyme of oxidative phosphorylation*

In a previous publication from this laboratory[1], it was shown that the heat-stable factor required for phosphorylation coupled to DPNH** oxidation in *Alcaligenes faecalis* extracts contained a polynucleotide of the RNA type. The activity remained with the polynucleotide during a number of purification procedures, but this did not exclude the possibility that a contaminant rather than the polynucleotide itself was active in the reaction. The obvious way to clarify this question seemed to be to test synthetic polynucleotides made by the enzyme polynucleotide phosphorylase[2]. It was also felt that a more rapid and delicate test for phosphorylation coupled to DPNH oxidation could be developed based on the spectrophotometric method of PULLMAN AND RACKER[3].

A test was developed which depends on using the ATP formed by oxidative phosphorylation to form TPNH according to the following reactions:

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{Hexokinase} + \text{Mg}^{++}} \text{G-6-P} + \text{ADP} \qquad (1)$$

$$\text{G-6-P} + \text{TPN} \xrightarrow{\text{G-6-P dehydrogenase}} \text{TPNH} + \text{6-PGA} \qquad (2)$$

It is different from PULLMAN AND RACKER'S test in that an oxygen electrode is not required, since the rate of oxidation is measured by the disappearance of DPNH in a control vessel which lacks some essential component required for oxidative phosphorylation or for the formation of TPNH. A second vessel containing the complete system gives the combined rate of DPNH disappearance and TPNH accumulation, and from the difference between the two, TPNH accumulation representing oxidative phosphorylation can be calculated. In some cases a third vessel (a DPN control) is used to measure any TPNH formed by pathways other than oxidative phosphorylation. The test is carried out in 1 ml Beckman cuvettes. All reagents except DPNH or DPN are added, readings are made at 340 mμ, and carefully measured known amounts of DPNH and DPN are then added to the appropriate vessels and readings are again made at intervals. Any TPNH accumulation found in the DPN vessel (the reading before DPN addition serves as the control) is obviously not the result of DPNH oxidation, and is therefore subtracted from the phosphorylation value derived from the first two vessels.

That this is a valid method of testing oxidative phosphorylation was shown by the fact that the test was dependent on the addition of TPN, hexokinase and G-6-P dehydrogenase, mononucleotide (either AMP or ADP), polynucleotide, and inorganic phosphate. By stopping the reaction and deproteinizing, it was possible to show inorganic phosphate uptake and, in the absence of TPN, G-6-P accumulation. $2 \cdot 10^{-4} M$ DNP inhibited phosphorylation up to 60% with this test, while $3.3 \cdot 10^{-4} M$ DNP produced 73% inhibition using the manometric method used previously[4].

Finally, MARTIUS AND NITZ-LITZOW[5] reported that menadione in 10^{-4} and $10^{-5} M$ concentration produced respectively 60 and 40% inhibition of oxidative phosphorylation in mitochondrial preparations. Using the spectrophotometric test and the *Alcaligenes faecalis* system these same concentrations of menadione gave respectively 64 and 36% inhibition of phosphorylation. In view of these findings the spectrophotometric test seems to be a valid one. P/O ratios as high as 0.7 have been found using it, which are significantly higher than the values found using the previous test.

To test the activity of a synthetic polynucleotide, 42.7 μmoles of ADP (Sigma chromatographed) were incubated with the soluble heat-labile fraction of *Alcaligenes faecalis* for 15 min at 30° C in TRIS buffer, pH 8.1, in the presence of magnesium chloride. The reaction was stopped by boiling, and the polynucleotide was recovered by precipitating with alcohol in the presence of magnesium chloride at pH 5.4. The recovered material was then dialyzed for two hours with stirring against cold water. A total of 14.1 μmoles of ADP were recovered in the polymerized state. This material was tested for its phosphorylating activity and compared with a sample of naturally occurring polymer purified by treatment with RNase and DNase and precipitation and dialysis as above, as well as with the sample of non-polymerized ADP. The results of one of several such experiments are shown in Table I.

It can be seen from this table that the synthetic polymer gives approximately 55% of the phosphorylation associated with the natural polynucleotide. There is also a small amount of activity in the non-polymerized ADP. Since the soluble heat-labile fraction used in the test contains large

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** The following abbreviations are used: DPN and DPNH for oxidized and reduced diphosphopyridine nucleotide; TPN and TPNH for oxidized and reduced triphosphopyridine nucleotide; RNA and DNA for ribose and desoxyribose nucleic acids; AMP, ADP and ATP for adenosine mono-, di-, and tri-phosphate; G-6-P for glucose-6-phosphate; 6-PGA for 6-phosphogluconic acid; DNP for 2,4-dinitrophenol; TRIS for tris(hydroxymethyl)aminomethane.

TABLE I

DEPENDENCE OF PHOSPHORYLATION ASSOCIATED WITH OXIDATION OF DPNH ON ADDITION OF NATURAL OR SYNTHETIC POLYNUCLEOTIDES

Addition	*Increased readings at 340 mμ caused by TPNH accumulation*		
	Vessel 1 Control – no polymer DPNH	*Vessel 2 Complete DPNH*	*Vessel 3 Complete DPN*
Purified natural polymer	None	0.460	0.002
		—0.002	
		0.458	
Synthetic AMP polymer	None	0.454	0.204
		—0.204	
		0.250	
Non-polymerized ADP	None	0.172	0.106
		—0.106	
		0.066	

Each 1 cc Beckman cuvette contained in a final volume of 1 cc the following: 50 μmoles of glycylglycine buffer pH 7.2, 0.5 μmoles of inorganic phosphate, 6 μmoles of $MgCl_2$, 25 μmoles of glucose, 0.05 μmoles of AMP, 0.5 μmoles of TPN, 37,500 RACKER units[6] of hexokinase, 0.123 KORNBERG units[7] of G-6-P dehydrogenase, particulate *Alcaligenes faecalis* fraction containing 176 DPNH oxidase units, and soluble fraction containing 66 units. In addition, one of the following was added as indicated: natural polynucleotide of the RNA type equivalent in absorption at 260 mμ to 0.52 μmoles of polymerized ADP, 0.52 μmoles of polymerized ADP, or 0.52 μmoles of unpolymerized ADP. After preliminary readings at 340 mμ, DPNH equivalent to a reading of 2.183 was added to each of the first two vessels and an equimolar amount of DPN to the third. The increments in readings at 340 attributable to TPNH accumulation are reported. TPNH accumulation in Vessel 3 is subtracted from that in Vessel 2, since it represents phosphorylation not associated with DPNH oxidation. It is presumably the result of depolymerization and formation of ATP by myokinase.

amounts of polynucleotide phosphorylase, it is not surprising that the mononucleotide has a little activity since some of it must be polymerized during the preliminary readings.

The previous variable results[1] obtained with AMP polymers supplied us by GRUNBERG-MANAGO which were tested with the old method, may be explained by depolymerization during the 5 min temperature equilibration period used in the manometric test before tipping in the DPNH. They may also have been the result of the large molecular weight of the polymers supplied by her which did not move on paper chromatography[8], while the AMP polymer used here had the same R_F (0.81) as the natural polymer when chromatographed on paper using *iso*amyl alcohol layered over 2% disodium phosphate.

Polymers made from uridine and cytidine diphosphates have also been found active in the phosphorylation reaction, in some cases more active than an equal amount of the naturally occurring polymer. These results make it extremely difficult to explain the action of the polynucleotides by a contaminant, since this hypothetical compound would have to come either from the enzyme forming the polynucleotide or from impurities in the mononucleotide used to make the polymers. Table I shows the latter assumption to be erroneous, and the nucleotides are polymerized with the same bacterial fraction that is used in the test, so this cannot be the source of the contaminant. This polynucleotide is not used up in the reaction. It is not only a new coenzyme of oxidative phosphorylation, but as a polynucleotide it is a new type of coenzyme.

Papers describing in more detail the work reported here and in a previous note are now in preparation.

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