THE ENZYMIC PHOSPHORYLATION OF MYO-INOSITOL*

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Myo-inositol is known to be an important precursor of polysaccharides and of ascorbic acid and other smaller metabolites (Loewus, 1963). A number of workers have demonstrated in cell-free systems the direct incorporation of free myo-inositol into myo-inositol monophosphatides (Paulus and Kennedy, 1960).

Loewus and co-workers (Loewus, Kelly and Neufeld, 1962; Loewus and Kelly, 1963) have obtained evidence that myo-inositol is oxidatively cleaved to yield glucuronic acid. Albersheim (1963) has presented data indicating that myo-inositol is converted into anhydrogalacturonic acid at the same rate that glucose and the methyl of methionine is incorporated into this polymeric galacturonide. He suggested that the three compounds pass through a common intermediate, presumably a methyl-galacturonate nucleotide.

Loewus (1964) recently proposed that methyl ether derivatives of inositols may be formed prior to oxidative cleavage of the cyclitol ring to yield directly methylated uronic acids. He suggests that the reactions might involve phosphorylated intermediates. Hoffmann-Ostenhoff et al (1958) demonstrated that crude yeast hexokinase phosphorylates myo-inositol. This paper will present evidence that an enzyme preparation which readily phosphorylates myo-inositol has little ability to phosphorylate glucose.

Experimental

Myo-inositol-U-C^{14} (36 mC/mM) was obtained from Nuclear

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Chicago Corp. and glucose-U-C^{14} (diluted to 36 mC/mM) was obtained from New England Nuclear Co. The triphosphate nucleotides, ATP, GTP, UTP, CTP and TTP, were purchased from Sigma Chemical Company.

The phosphorylating enzyme was prepared from germinated mung bean seeds (Phaseolus aureus) by the procedure of Neufeld et al (1961) except that Tris-maleate buffer (pH 7.5) was used throughout. Ammonium sulfate fractions were obtained at 0-40%, 40-50%, 50-55% and 55-70% of saturation. Enzyme extracts were prepared similarly from sycamore cells in liquid culture (Acer pseudoplatanus), from Escherichia coli and from bovine liver.

A typical enzyme assay contained the following solutions: 10 μl of ATP or other nucleotide (0.015 M), Mg^{++} (0.04 M), NaF (0.032 M), mercaptoethanol (0.1 M), Tris-maleate buffer (0.1 M, pH 7.5); 5 μl of 1.4×10^{-4} M myo-inositol-U-C^{14}, and 5 μl of the mung bean extract.

Enzyme assays were carried out in sealed glass capillary tubes. The reaction was stopped either by freezing, or by simply applying the reaction mixture to Whatman No. 1 filter paper. Product was separated by high voltage electrophoresis in 0.05 M ammonium formate buffer, pH 3.6. Electrophoresis was carried out for two hours with 100-120 V/cm at 5-10°C. The myo-inositol and other neutral compounds moved approximately 1/2 inch in the direction of the positive pole. Myo-inositol phosphate moved in the same direction a distance of 8 to 9 inches; approximately 0.9 to 1.0 times the distance traveled by picric acid. Radioactive compounds were located with the aid of a radiochromatogram scanner. In most cases the actual radioactivity was determined by eluting the compounds and counting them on planchets. The data of the kinetic studies (see Figure 1) was obtained directly from the electrochromatograms with the aid of an electronic integrator connected to the radiochromatogram scanner.

Reducing sugars were identified by dipping the filter papers in the amino-biphenyl reagent of Gordon et al (1956). Inositol was detected by the $AgNO_3$ method of Trevelyan (1950). Phosphate esters were detected by the method of Hanes and Isherwood (1949).

Results and Discussions

The formation of the phosphorylated product is linear under the conditions used for the first forty minutes (see Figure 1). Synthesis then levels off sharply and, indeed, the amount of product declines slowly. The results of Figure 1 are the average of two experiments.

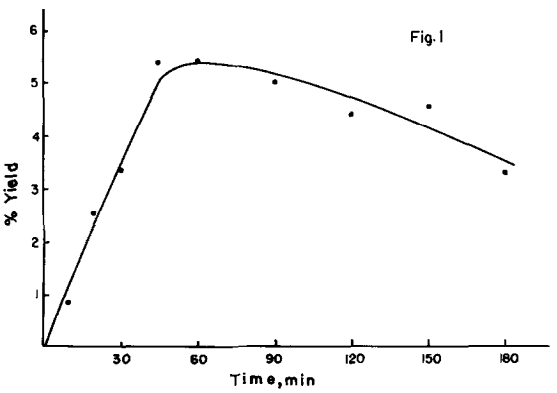

Figure 1. Synthesis of myo-inositol phosphate at 23°C. Complete reaction mixture: 0.15 μM ATP, 0.4 μM Mg^{++}, 0.32 μM NaF, 1 μM mercaptoethanol, 1 μM Tris-maleate (pH 7.5), 5 x 10^4 dpm of myo-inositol-U-C^{14}, and 5 μl of enzyme solution.

The maximum yield obtainable under these conditions is about 20% of the myo-inositol introduced into the reaction mixture. Such yields were obtained in experiments designed to determine the pH optimum of the enzyme (Figure 2). The pH curve is the average of two experiments in which reaction mixtures were incubated for two hours at 23°C in 0.05 M Tris-maleate buffer.

Identification of the product as myo-inositol phosphate was achieved by electrophoresis in ammonium formate buffer, by chromatography in acetic acid-ethyl acetate-water (2.5:5:3), and by hydrolysis of the product to yield free myo-inositol. It is well known that myo-inositol phosphate is hydrolyzed with great difficulty (McCormick and Carter, 1952). The product formed by enzyme reaction was not demonstrably hydrolyzed in 1 N HCl at 94°C for one hour. Only 37% of the product was hydrolyzed in 1 N HCl at 94°C for fourteen hours, while 78% of the product was hydrolyzed in 6 N HCl at 94°C for sixteen hours. This

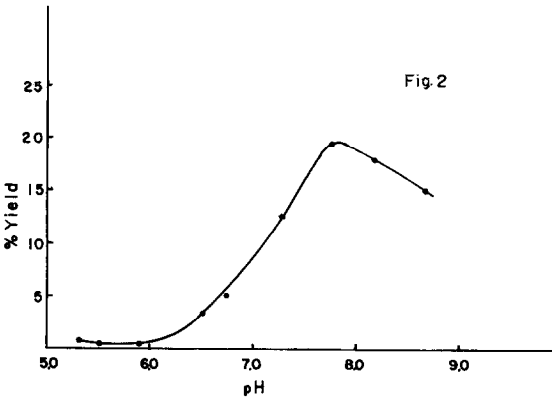

Figure 2. pH dependence of myo-inositol kinase activity. Same reaction conditions as Figure 1 except that pH was as indicated.

severe hydrolysis yielded only two products, a minor peak which migrated during electrophoresis and chromatography as the original product and a major peak which moved during electrophoresis in ammonium formate buffer and chromatography in acetic acid-ethyl acetate-water as myo-inositol. The myo-inositol thus liberated was further identified by electrophoresis in 0.05 M borate buffer (pH 9.2). The product of hydrolysis migrated in the borate system in a manner similar to that of myo-inositol.

The mung bean enzyme extract phosphorylates myo-inositol more readily than it does glucose. In a typical experiment, 6900 counts per minute of myo-inositol phosphate were formed as compared to 460 counts per minute of a compound which migrated during electrophoresis as phosphorylated glucose. This experiment was carried out under conditions in which the concentration and radioactivity of myo-inositol and glucose were nearly identical.

The specificity of the enzyme for nucleoside triphosphate is not great. It was possible to phosphorylate myo-inositol with either ATP, GTP, or UTP. CTP had little activity, and TTP essentially none (see Table 1). This lack of nucleotide specificity is not unusual (see, for instance, Neufeld et al, 1960).

A product which migrated at the same rate as myo-inositol phosphate could be obtained with enzyme extracts from sycamore cells in culture, from Escherichia coli, and from bovine liver. The most active preparations were found in all cases in the 40-50% saturated $(NH_4)_2SO_4$ fraction. The 50-55% $(NH_4)_2SO$ fraction contained a slight amount of enzymic activity. The particular (mitochondrial) fraction from all sources failed to yield an active enzyme.

TABLE 1

Nucleotide Requirement for Phosphorylation of Myo-Inositol

Nucleotide Added	Product CPM
ATP	4720
GTP	3180
UTP	2540
TTP	560
NONE	400

Attempts to couple the kinase catalyzed reaction with the oxidation of NADH by addition to the reaction mixture of phosphoenolpyruvate, pyruvate kinase, NADH, and lactic dehydrogenase failed because of the presence in the enzyme mixture of an ATPase. Efforts were made to inhibit the ATPase with fluoride ion. It was, however, not possible to reduce the background level of ATPase sufficiently to assay for myo-inositol kinase. We are currently attempting to obtain sufficiently purified myo-inositol kinase for use in such an assay system.

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