Myoinositol Kinase: Partial Purification and Identification of Product

Abstract. *Myoinositol kinase found in plant, animal, and microbial extracts has been partially purified by density-gradient centrifugation. The product of the enzymic reaction has been tentatively identified by paper chromatography as myoinositol-1-phosphate.*

Myoinositol is an important precursor of cell wall polysaccharides (*1*). The initial step in the pathway to polysaccharide synthesis may be the conversion of myoinositol to myoinositol-6-phosphate. Myoinositol-6-phosphate might then react with a nucleoside triphosphate to form a nucleoside diphosphate myoinositol, for example, uridine diphosphate (UDP) myoinositol. The inositol ring could then be cleaved oxidatively to yield UDP glucuronic acid, a known intermediate in the pathway to polysaccharide synthesis (*2*).

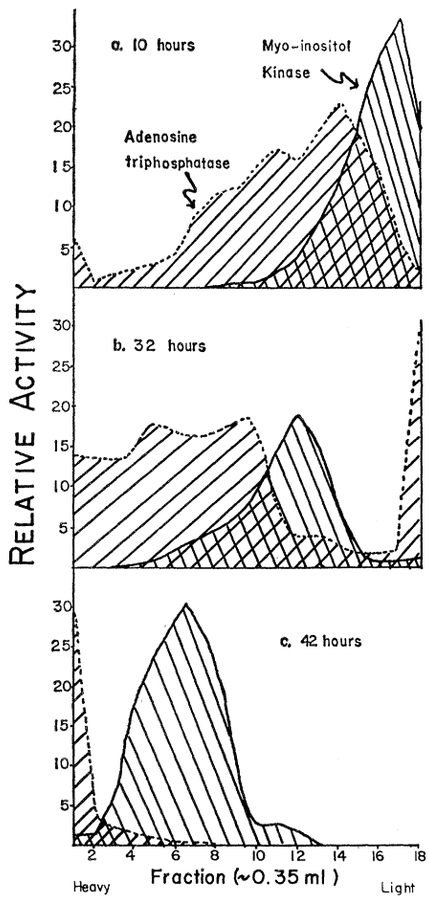

Fig. 1. Separation of mung-bean myoinositol kinase from adenosine triphosphatase by density-gradient centrifugation. Effect of length of centrifugation on migration is shown in parts *a*, *b*, and *c*. Relative kinase activity is a measure of amount of C^{14}-labeled product formed by enzymic catalysis; relative adenosine triphosphatase activity is a measure of rate of oxidation of NADH in a coupled reaction.

An enzyme which catalyzes the phosphorylation of myoinositol was found in extracts prepared from germinated mung-bean seeds (*3*). Attempts were made to assay the enzyme spectrophotometrically by measuring adenosine diphosphate (ADP) produced in the reaction between myoinositol and adenosine triphosphate (ATP). These were unsuccessful due at least in part to the presence of an adenosine triphosphatase in the enzyme extract.

The presence of myoinositol kinase in extracts from sycamore cells grown in culture, from *Escherichia coli*, and from bovine liver has been reported previously (*3*). We have now found kinase activity in enzyme extracts prepared from other sources. The method of preparation is similar to that used for the mung-bean extract (*3*). Kinase activity is present in the 40 to 60 percent saturated $(NH_4)_2SO_4$ fraction of ground fresh spinach leaves. Substantial kinase activity is found, too, in the 30 to 50 percent saturated $(NH_4)_2SO_4$ fraction of fresh wheat germ that had been soaked previously for 30 to 40 minutes in cold sucrose solution. A highly active adenosine triphosphatase is present in both preparations. The wheat germ extract, in contrast with the mung-bean extract, can be frozen and lyophilized with no loss of myoinositol kinase activity.

Separation of myoinositol kinase from adenosine triphosphatase was attempted by ion-exchange chromatography on diethylaminoethyl- and on carboxymethylcellulose. Although substantial purification could be achieved, most of the activity of the preparation was lost irreversibly. The two enzymes were not separable by gel filtration on various preparations of Bio-Gel or Sephadex. However, the kinase and adenosine triphosphatase were separated by density-gradient centrifugation. Enzyme solution (0.4 ml) prepared from mung bean (*3*) was layered on a sucrose density gradient (5 to 20 percent) containing 0.025*M* tris maleate buffer, *p*H 7.7, and 0.025*M* 2-mercaptoethanol. Centrifugation was carried out in a Spinco SW 39L rotor at 39,000 rev/min at 2°C. The bottom of the centrifuge tube was punctured, and the contents were collected as fractions of approximately 0.35 ml.

Each fraction was assayed for myoinositol kinase by enzymically phosphorylating radioactive myoinositol (*3*). The phosphorylated product was separated by high-voltage electrophoresis. Relative activity was obtained from the digital "readout" of an electronic integration of the product peak as detected by a radiochromatogram scanner. All fractions were also assayed for adenosine triphosphatase by coupling the enzyme-catalyzed reaction with the oxidation of reduced nicotinamide adenine dinucleotide (NADH) (*4*). The reaction mixture was as follows: $MgCl_2$ (6.7 mg), phosphoenolpyruvic acid (5.5 mg), ATP (4 mg), KCl (3 mg), lactate dehydrogenase (7 μl, Sigma type I, containing 43 mg of protein per milliliter), pyruvate kinase (5 μl, Sigma Type II, containing 10 mg of protein per milliliter), and NADH (1.0 mg). This mixture was dissolved and adjusted to *p*H 7.5 with dilute KOH and diluted to 0.4 ml with distilled water. Ten microliters of this mixture, 20 μl from each of the fractions obtained from the density gradient, and 0.3 ml of 0.025*M* tris maleate buffer, *p*H 7.7, containing 0.025*M* 2-mercaptoethanol were mixed in a cuvette, and the conversion of NADH to NAD was followed on a Gilford spectrophotometer at 340 mμ at 25°C. Relative activity was obtained from the rate of oxidation of

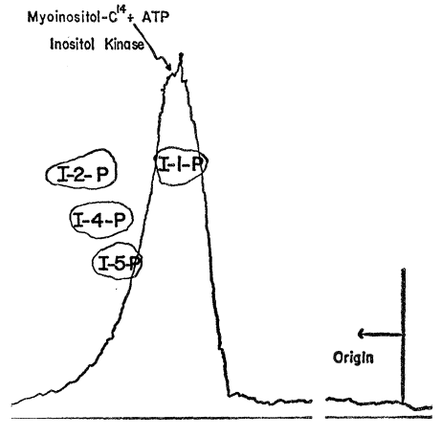

Fig. 2. Descending co-chromatography of the C^{14}-labeled phosphorylated product of myoinositol kinase and authentic unlabeled isomers. Myoinositol-1-phosphate (I-1-P) migrated 18.5 cm; myoinositol-5-phosphate (I-5-P), 20.3 cm; myoinositol-4-phosphate (I-4-P), 20.9 cm; and myoinositol-2-phosphate (I-2-P), 21.9 cm. The kinase product migrated 18.5 cm. The solvent front was 47.5 cm from the origin.

NADH. The result of such assays after 10, 32, and 42 hours of centrifugation is illustrated in Fig. 1, *a–c*, respectively. The best separation of myoinositol kinase and adenosine triphosphatase was achieved after 42 hours.

We were not successful in coupling the purified myoinositol-kinase–catalyzed reaction with the oxidation of NADH. The assay solutions inhibited myoinositol kinase.

Identification of the particular isomer of myoinositol phosphate formed by the enzyme-catalyzed reaction was achieved by descending chromatography in a mixture of isopropyl alcohol, ammonia, and water (70:10:20) at 40°C for 32 hours (*5*). Radioactive myoinositol phosphate was eluted from electrophoretograms and treated with activated charcoal to remove nucleotides. The product was then mixed with each of the possible isomers of myoinositol phosphate (the 1-, 2-, 4- and 5-) and spotted on Whatman No. 1 chromatography paper. The 1- and 3-, and 4- and 6- isomers are D,L pairs and are indistinguishable by our techniques. The chromatograms were analyzed with a radiochromatogram scanner to locate the radioactive product which had been mixed with an authentic unlabeled isomer. Each chromatogram was then sprayed with Hanes-Isherwood reagent to locate the phosphate portion of the unlabeled isomer (*6*). The product of myoinositol kinase was found to migrate as myoinositol-1-phosphate and was separated from the other isomers (see Fig. 2).

Verification of the identity of the enzymic reaction product was carried out by the following procedure: Myoinositol-2-phosphate (Cal Biochem) was submitted to weak acid hydrolysis (1*N* HCl at 80° to 85°C for 30 minutes) to yield an equilibrium mixture of the 1- and 2- phosphates (*7*). The hydrolysis solution was neutralized with 1*N* NH_4OH, mixed with radioactive product and chromatographed as described. Myoinositol-1- and -2-phosphates were run as standards. The location of the radioactive product again corresponded to that of the 1-phosphate and was separated completely from the 2-phosphate.

Tentative identification of the product as myoinositol-1-phosphate opens the question of whether this enzymic reaction is the initial step in polysaccharide synthesis. It seems unlikely that the 1-phosphate (which would result in formation of a sugar-6-phosphate) would be in this pathway. This reaction may be the initial step in synthesis of phytic acid, the hexaphosphate ester of myoinositol, or, perhaps it has some other as yet unknown function.

PATRICIA D. ENGLISH
MICHAEL DIETZ
PETER ALBERSHEIM
Department of Chemistry,
University of Colorado, Boulder

References and Notes

1. F. A. Loewus, *Phytochemistry* **2**, 109 (1963).
2. D. S. Feingold, E. F. Neufeld, W. Z. Hassid, in *Modern Methods of Plant Analysis*, H. F. Linskens and M. J. Tracey, Eds. (Springer, Berlin, 1964), vol. 5, p. 2.
3. M. Dietz and P. Albersheim, *Biochem. Biophys. Res. Commun.* **19**, 598 (1965).
4. A. Kornberg and W. E. Pricer, *J. Biol. Chem.* **193**, 481 (1951).
5. F. L. Pizer and C. E. Ballou, *J. Amer. Chem. Soc.* **81**, 915 (1959).
6. C. S. Hanes and F. A. Isherwood, *Nature* **164**, 1107 (1949).
7. S. J. Angyal and M. E. Tate, *J. Chem. Soc.* **1961**, 4128 (1961).
8. Supported in part by NIH research grants GM12655 and GM12656, by AEC contract AT(11-1)-1426, and by funds provided by the Council on Research and Creative Work, University of Colorado. We thank Dr. C. E. Ballou, University of California, Berkeley, for authentic samples of myoinositol phosphates.

4 October 1965

Artificial Reestablishment of the Lichen Cladonia cristatella

Abstract. *Slow drying stimulated the artificial development of fruiting structures by* Cladonia cristatella *Tuck. Fructification of the fungal partner occurred in the absence of its algal symbiont. Only newly isolated mycobionts produced the reproductive structures. Soredia and squamules were formed in response to the joint effect of slow drying and nutrient-poor conditions.*

Perhaps the most puzzling problem in lichenology has been the inability to reconstitute a lichen association in laboratory culture. Although considerable progress has been made in this direction (*1*), the final step in the lichen development sequence (*2*), that is, fruiting of the lichen fungus, has never been attained. The persistent record of failure in attempts to achieve this stage has hampered progress in both experimental and descriptive lichenology and has raised doubts as to whether the separate symbionts still retain the capacity to reestablish well-defined lichenized associations.

The experimental procedure for the first part of my investigation was as follows: Spores from the lichen's fruiting bodies (apothecia) were allowed to discharge into 250-ml erlenmeyer flasks which contained either soil-extract or malt-yeast-extract agar (*3*). One set of flasks contained only the agar medium. In another set each flask contained, in addition to the agar medium, a fragment of rotted wood, apple or maple, which was taken from the original substrate on which some of the test specimens were growing. The wood fragments were moistened with distilled water and placed either horizontally on the bottom of the flask or in a slanted position. The flasks with the wood were autoclaved and kept for 24 hours at 37°C to allow for germination of any viable contaminant spores on the wood. Medium was then added to the flasks to a level which would not fully cover the surface of the wood, and the flasks were autoclaved again. Each of the two sets of flasks was divided into two groups. One group received a suspension of the alga *Trebouxia erici* Ahm. (*4*), a phycobiont of *Cladonia cristatella* which has been maintained in culture for 7 years, and the other group received only the suspensory medium (*5*) and not the alga. Spore discharge into the flasks was accomplished by placing washed apothecia from the lichen onto petri dish covers, by means of vaseline, and placing these covers over the mouths of the flasks. The covers were kept in this position for as long as 2 days, or until a sufficient spore discharge had been obtained. One of the covers with apothecia was placed over the bottom of a petri dish filled with agar. By examining the dish at periodic intervals, I could determine the relative amount of spore discharge from the population of lichen which was being used. The covers then were replaced with sterile cotton plugs and the flasks were incubated at 20°C. After germination of the spores, which again could be determined by examining the "control" petri dish, a suspension of the alga from a young, liquid culture was added to one set of the flasks. All flasks were kept under constant illumination at a light intensity of about 1100 lu/m^2.

After 2 weeks, the alga formed a heavy, continuous growth over the agar surface and parts of the wood fragments. Scattered colonies of the